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HEPATIC GENE NETWORKS DUE TO THE TRANS-10, CIS-12-CONJUGATED
LINOLEIC ACID SUPPLEMENTATION IN GROWING MALE MICE

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Nutritional Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2011

Urbana, Illinois

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ABSTRACT

Trans-10, *cis*-12-conjugated linoleic acid (*trans*-10, *cis*-12-CLA) has been reported to reduce body fat mass but accompanied by increased liver mass. However, the cellular and molecular mechanisms have been unknown. Our study investigated the effects of *trans*-10, *cis*-12-CLA supplementation on hepatic gene expression in growing male mice by measuring mRNA abundance of thirty-eight genes encoding important enzymes, ligand-dependent nuclear receptors, transcription regulators and transporters in lipid metabolism. Feeding growing male mice diets supplemented with 0.3% *trans*-10, *cis*-12-CLA for 6 weeks significantly increased the expression of hepatic genes involved in fatty acid uptake (Cd36), TG synthesis (Acaca, Gpam and Scd), lipid droplet formation (Plin2), VLDL assembling/secretion (Mttp and Cideb), ketogenesis and utilization (Hmgcs2 and Bdh1), fatty acid oxidation (Acox1), glyceroneogenesis (Pck1 and Pdk4). The results suggest that the increased liver weight in response to *trans*-10, *cis*-12-CLA may be due to increased fatty acid uptake, stimulated TG synthesis, enhanced formation of lipid droplets as well as insufficiently induced VLDL assembly/secretion mechanism in liver. We provided new evidences to advance in the knowledge of the effect of this supplement in a relatively low amount on hepatic gene expression in growing male mice.

ACKNOWLEDGMENTS

I wish to express my gratitude to all the people who helped me in making this research project a reality. Thanks to my advisor, Assistant Professor Juan J. Loor, for his constant support and guidance through the highs and the lows of my development as a scientist, colleague and lifelong learner. Also thanks to my committee members, Professor James K. Drackley, Professor Walter L. Hurley and Assistant Professor Yuan-Xiang Pan, who offered valuable comments and trustworthy counsel.

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CHAPTER 1

Introduction

The World Health Organization (WHO) reports that the global epidemic of overweight and obesity – “globesity” – is rapidly becoming a major public health problem in many parts of the world (1). Over the past two decades, the incidence of overweight and obesity has rapidly increased in industrialized countries and currently affects about 72.3% of male and 64.1% of female adults in the U.S. (2). Meanwhile, the most recent National Health and Nutrition Examination Survey (NHANES) shows that 16.9% of children ages 2-19 are obese and 31.7% are overweight (3). Besides, the medical care costs ascribed to obesity and associated disorders accounted for around \$168 billion (4) in the U.S., thus constituting a substantial economic burden. In the course of this alarming epidemic trend, so-called functional foods that match both the growing health awareness and need for convenience attract much attention (5), such as conjugated linoleic acid (CLA).

CLA refers to a group of conjugated octadecadienoic acid isomers derived from linoleic acid (LA), a fatty acid (FA) that contains 18 carbons and 2 double bonds (6). CLA isomers are typically found in the products of ruminant animals, such as meat and milk, which arises in their gastrointestinal tract where microbes convert LA into different isoforms of CLA through biohydrogenation (7). This process changes the position and configuration of the double bonds, resulting in a single bond between one or both of the two

double bonds, i.e., *trans*-10, *cis*-12-CLA or *cis*-9, *trans*-11-CLA.

Experiments conducted using pure synthetic *trans* -10, *cis*-12-CLA have shown that this isomer might have beneficial effects against obesity (8), diabetes (9), atherosclerosis (10), and cancer (11). The anti-lipogenic effects of dietary CLA mixtures of *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA with a ratio of 1:1, were initially observed in several lines of mice, and later work using purified isomer preparations clearly showed that *trans*-10, *cis*-12-CLA was responsible for delipidation (12-16). Supplementation with *trans*-10, *cis*-12-CLA decreases body fat mass in many animal studies and some human studies (41, 42). Furthermore, some previous reports suggest that dietary supplementation with CLA reduces body fat mass in overweight humans without disclosing any adverse effects (17, 18), indicating that *trans*-10, *cis*-12-CLA may be considered as a weight loss agent. Therefore, CLA supplements containing *trans*-10, *cis*-12-CLA (e.g., Your Life[®], Natrol[®] and Nature's Way[®], Clarinol[™], Met-Rx, Dymatize[®], EAS[®]) are readily available in Health Food Stores and stores where nutritional supplements are sold.

However, recent studies revealed unfavorable metabolic effects of *trans*-10, *cis*-12-CLA associated with increased oxidative stress and elevated inflammatory biomarkers (19, 20). They have found that *trans*-10, *cis*-12-CLA indeed could considerably reduced body fat mass but accompanied by hepatic steatosis (5, 12, 21-25) which is an excess accumulation of triacylglycerol (TG) in the liver (26) and caused by an imbalance

between lipid availability (from circulating lipid uptake or de novo lipogenesis) and lipid disposal (via fatty acid oxidation or TG-rich lipoprotein secretion) (27). As *trans*-10, *cis*-12-CLA supplementation reduces a amount of adipose tissue, several studies suggest that much of the increased liver mass may be explained by accumulation of lipids (28, 29) derived from mobilization of delipidated adipose tissue stores (30, 31).

The hepatic steatotic effect of *trans*-10, *cis*-12-CLA has been considered to be related to modulate the expressions of genes relevant to energy expenditure, apoptosis, fatty acid oxidation, lipolysis, differentiation and lipogenesis (6, 32). A study conducted by Clément et al. showed that mice fed a diet enriched in *trans*-10, *cis*-12-CLA (0.4% w/w) for 4 weeks developed lipoatrophy, hyperinsulinemia and fatty liver (33). In this study, they investigated the levels of expression of genes encoding transcription factors, lipid-binding proteins and enzymes known to play a significant role in lipid metabolism (33). Finally, the results suggest that liver steatosis might be triggered by *trans*-10, *cis*-12-CLA-induced hyperinsulinemia, by inducing both fatty acid uptake and lipogenesis as evidenced by the substantial up-regulation of peroxisome proliferator activated receptor γ (Ppar γ), Cd36, fatty acid binding protein 4 (Fabp4) and sterol regulatory element binding transcription factor 1 (Srebf1) (33). However, the cellular and molecular mechanisms by which *trans*-10, *cis*-12-CLA results in hepatic steatosis in mice have been unclear.

To our knowledge, there are no published studies dealing with the genomic effects

of chronic dietary *trans*-10, *cis*-12-CLA supplementation on liver of growing animals that could serve as models for humans. In our previous study (24), relatively low amount of *trans*-10, *cis*-12-CLA (0.30%) was used compared to other studies (22, 23, 28, 32, 34-36). We observed that dietary *trans*-10, *cis*-12-CLA resulted in a linear increase in liver weight as well as a reduction in adipose tissue weights in both male and female mice over the 6-week feeding period, which substantiated some published findings obtained at higher amounts of CLA (i.e., 0.5-1% of the diet) (28, 37). The present study utilized growing male mice which are more susceptible than females (24) to investigate the effects of *trans*-10, *cis*-12-CLA on hepatic gene expression. Our hypothesis was that long-term increased liver mass in response to *trans*-10, *cis*-12-CLA would be associated with greater mRNA abundance of lipogenic gene networks (Figure 1.1). Specific objectives were to measure mRNA abundance of thirty-eight genes (Table 1.1) encoding proteins required for FA uptake, FA oxidation, de novo FA synthesis, lipolysis, lipogenesis, ketogenesis, carbohydrate metabolism, inflammation, stress response, growth factor signaling, transcriptional regulation of lipogenesis and lipid droplet formation.

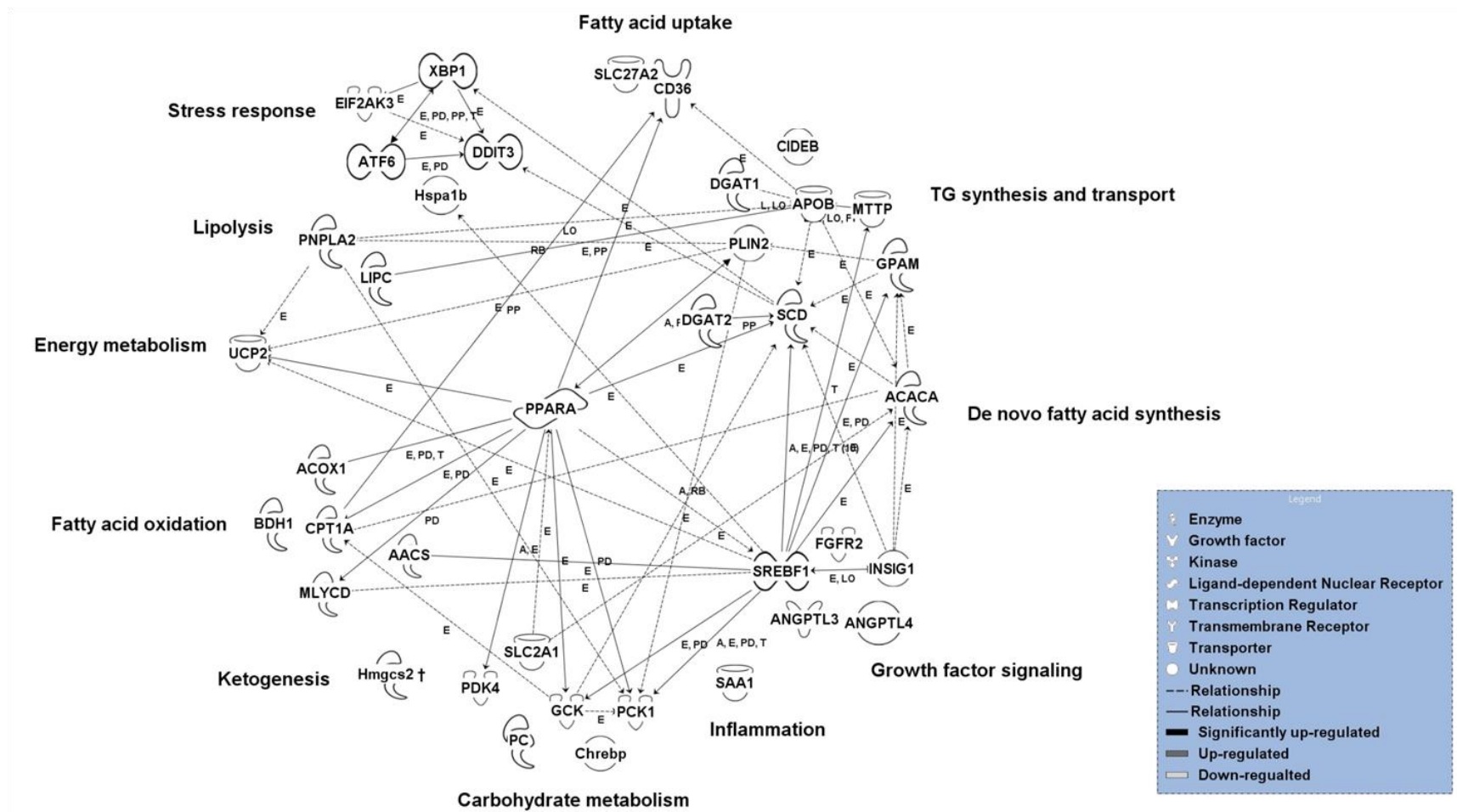


Figure 1.1 Currently known relationships among genes analyzed based on manually curated examination of the published literature within the Ingenuity Pathway Analysis (www.ingenuity.com) knowledge base. Genes are grouped by the predominant process they play in lipid metabolism. Different shapes denote the type of protein encoded by the specific genes, including enzymes, ligand-dependent nuclear receptors, transcription regulators, and transporters. Letters along the edges denote effects on activity (A), expression (E), localization (LO), proteolysis (L), RNA binding (RB), protein-DNA binding (PD), and protein-protein binding (PP). Gene names are as in Table 1.1.

Table 1.1 Gene symbol, description, location, biological process and overall % mRNA abundance among genes measured

Gene symbol	Description	Localization	Biological process¹	% RNA²
<i>Aacs</i>	Acetoacetyl-CoA synthetase	Cytoplasm	FA metabolism	0.57
<i>Acaca</i>	Acetyl-Coenzyme A carboxylase alpha	Cytoplasm	FA biosynthesis	0.52
<i>Acox1</i>	Acyl-Coenzyme A oxidase 1	Cytoplasm, peroxisome, mitochondrion	FA oxidation	6.83
<i>Angptl3</i>	Angiopietin-like 3	Extracellular space	FA metabolism	1.47
<i>Angptl4</i>	Angiopietin-like 4	Extracellular space	TG homeostasis	0.06
<i>Apob</i>	Apolipoprotein B	ER, microsome	Lipoprotein metabolism	9.14
<i>Atf6</i>	Activating transcription factor 6	Nucleus	Transcription regulation	0.02
<i>Bdh1</i>	3-hydroxybutyrate dehydrogenase 1	Mitochondrion	Oxidation-reduction process	3.35
<i>Cd36</i>	CD36 molecule (thrombospondin receptor)	Golgi, cell surface, mitochondrion	FA transport	0.00
<i>Chrebp</i>	MLX interacting protein-like	Cytoplasm, nucleus	Glucose homeostasis, transcription regulator activity	2.83
<i>Cideb</i>	Cell death-inducing DNA fragmentation factor alpha subunit-like effector B	Cytosol	Apoptosis	3.46
<i>Cpt1a</i>	Carnitine palmitoyltransferase 1a	Mitochondrion, microsome	FA metabolism	1.91
<i>Ddit3</i>	DNA-damage inducible transcript 3	Cytoplasm, nucleus	Cell cycle, transcription regulation	0.06
<i>Dgat1</i>	Diacylglycerol O-acyltransferase 1	ER, membrane	TG biosynthesis, VLDL assembly	0.15
<i>Dgat2</i>	Diacylglycerol O-acyltransferase 2	ER, membrane	TG biosynthesis, glycerol metabolism	4.93
<i>Eif2ak3</i>	Eukaryotic translation initiation factor 2 alpha kinase 3	Cytoplasm, ER	Srebp-mediated signaling pathway, apoptosis	0.03

Table 1.1 (cont.)

Gene symbol	Description	Localization	Biological process¹	% RNA²
<i>Fgfr2</i>	Fibroblast growth factor receptor 2	Nucleus, cell surface	Cell-cell signaling	0.05
<i>Gck</i>	Glucokinase	Cytoplasm, mitochondrion, nucleus	Carbohydrate phosphorylation	0.82
<i>Gpam</i>	Glycerol-3-phosphate acyltransferase, mitochondrial	Mitochondrion, membrane	TG biosynthesis, FA hemeostasis	3.18
<i>Hmgcs2</i>	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	Mitochondrion	Ketogenesis	13.88
<i>Hspa1b</i>	Heat shock 70kDa protein 1B	Cytoplasm, mitochondrion, nucleus	DNA repair, anti-apoptosis	0.11
<i>Insig1</i>	Insulin induced gene 1	Endoplasmic reticulum (ER)	Cholesterol biosynthesis, TG metabolism	4.14
<i>Lipc</i>	Lipase, hepatic	Cell surface, extracellular space	FA metabolism, TG metabolism, LDL clearance, HDL remodeling	3.92
<i>Mlycd</i>	Malonyl-CoA decarboxylase	Cytoplasm, mitochondrion, peroxisome	FA biosynthesis & oxidation	0.50
<i>Mttp</i>	Microsomal triglyceride transfer protein	Golgi, ER	Lipoprotein transport, TG metabolism	1.24
<i>Pcx</i>	Pyruvate carboxylase	Mitochondrion	Gluconeogenesis, lipid biosynthesis	1.42
<i>Pck1</i>	Phosphoenolpyruvate carboxykinase 1	Cytoplasm, nucleus	Gluconeogenesis, lipid metabolism, glycerol biosynthesis	14.79
<i>Pdk4</i>	Pyruvate dehydrogenase kinase, isoenzyme 4	Mitochondrion	Glucose metabolism	0.13
<i>Plin2</i>	Perilipin 2	Cytoplasm, nucleus, plasma membrane	Lipid storage, long-chain FA transport	1.61

Table 1.1 (cont.)

Gene symbol	Description	Localization	Biological process ¹	% RNA ²
<i>Pnpla2</i>	Patatin-like phospholipase domain containing 2	Cytosol, membrane	Lipid catabolic process	0.33
<i>Ppara</i>	Peroxisome proliferator activated receptor alpha	Nucleus	FA metabolism, glucose metabolism, transcription	1.28
<i>Saa1</i>	Serum amyloid A 1	Extracellular space	Cholesterol metabolism	0.54
<i>Scd</i>	Stearoyl-Coenzyme A desaturase 1	ER membrane	FA biosynthesis	2.53
<i>Slc27a2</i>	Solute carrier family 27 member 2	ER, microsome, peroxisome	FA transport, lipid metabolism	3.63
<i>Slc2a1</i>	Solute carrier family 2 member 1	Cytoplasm, plasma membrane	Glucose transport	0.07
<i>Srebf1</i>	Sterol regulatory element binding factor 1	Golgi, ER, nucleus	Transcription regulation	0.67
<i>Ucp2</i>	Uncoupling protein 2	Membrane, mitochondrion	Mitochondrion transport, response to superoxide	0.19
<i>Xbp1</i>	X-box binding protein 1	Nucleus	Transcription	9.67

¹ Entrez Gene, National Center for Biotechnology Information (NCBI).

² The % mRNA abundance is calculated by $[(1/E^{\Delta C_t}) \text{ specific gene} / \sum (1/E^{\Delta C_t}) \text{ all genes}] \times 100$. See Experimental Methods for details

CHAPTER 2

Literature review

Currently, more than two-thirds of American adults and nearly one-third of children and teens are either overweight or obese (2). Since 1980, the number of obese adults has doubled (38). Since 1970, the number of obese children ages 6-11 has quadrupled, and the number of obese adolescents ages 12-19 has tripled (38, 39). Obesity-related medical costs are nearly 10 percent of all annual medical spending (40). The alarming increases in obesity rates over the past two decades has attracted much attention to the anti-lipogenic effects of the *trans*-10, *cis*-12-CLA. Supplementation with *trans*-10, *cis*-12-CLA has been reported to reduce body fat mass in many animal studies and some human studies (41, 42). Besides, CLA supplements containing *trans*-10, *cis*-12-CLA are readily available in Health Food Stores and stores where nutritional supplements are sold. Although *trans*-10, *cis*-12-CLA seems to be a promising substance as regards reduction of body fat, it is important to consider the possibility of adverse effects found in animal models (43, 44) and more recently in specific groups of humans (45-47). The most recent studies have stated that *trans*-10, *cis*-12-CLA could cause hepatic steatosis in mice (5, 12, 21-25, 33). However, fatty liver formation due to *trans*-10, *cis*-12-CLA has not been reported in human as seen in mice. As CLA has been approved by FDA in GRAS category, considering the possible adverse effects of long-term use of CLA, especially *trans*-10, *cis*-12-CLA

isomer, further and more studies in animals and humans are urgently needed before the *trans*-10, *cis*-12-CLA usages as a dietary supplement to reduce obesity in humans (48).

This chapter will begin with the introduction of CLA, focusing on *trans*-10, *cis*-12-CLA isomer. Then it will discuss the physiological effects of *trans*-10, *cis*-12-CLA on adipose tissue and liver by reviewing *in vitro*, *in vivo* and clinical studies. Finally, it will summarize proposed underlying mechanisms through which *trans*-10, *cis*-12-CLA isomer exhibits its hepatic steatotic effect.

Definition of *trans*-10, *cis*-12-CLA

CLA is a fatty acid belonging to the group of polyunsaturated fatty acids (PUFAs) (49) as well as a generic term for the geometric and positional isomers of LA (*cis*-9, *cis*-12, octadecadienoic acid) (50) which contains 18 carbons and 2 double bonds. But, there is no methylene group separating the double bonds of CLA as there is in LA (51). Instead, the two double bonds are separated by one single bond (51) and are present predominantly in positions of carbons 9 and 11 (*cis*-9, *trans*-11-CLA), and 10 and 12 (*trans*-10, *cis*-12-CLA), which are two most studied CLA isomers (Figure 2.1).

CLA is formed in the gastrointestinal tract of ruminant animals where microbes convert LA into different isoforms of CLA through incomplete biohydrogenation (49), resulting in a single bond between one or both of the two double bonds (52). But, it has

been demonstrated that CLA can be also endogenously synthesized from vaccenic acid by Δ^9 -desaturase in rumens of lactating cows (53) and in lactating women (54). Therefore, natural forms of CLA can be found predominantly in ruminant food products, such as milk, cheese and beef (55-57), and present primarily (90%) as *cis*-9, *trans*-11-CLA (52, 53). According to Atkinson, the amount of CLA obtained by foods is 200 mg/day (58); Chin et al. estimated that the daily intake of CLA in humans is approximately 160 mg (59) and Ritzenthaler et al. suggested 212 mg in men and 151 mg in women (60). Hence, in order to obtain the beneficial effects of CLA on health, it is necessary to include an additional supplementation of CLA in the habitual diet in view of the amount and type of isomer consumed habitually (49).

Commercial preparations of CLA supplements are synthesized by alkaline isomerization of LA-enriched vegetable oils (e.g. safflower and sunflower oil) (61). This type of processing yields a CLA mixture containing approximately 40% of the *cis*-9, *trans*-11-CLA isomer and 44% of the *trans*-10, *cis*-12-CLA isomer (16). Commercial preparations also include about 4-10% *trans*-9, *trans*-11-CLA and *trans*-10, *trans*-12-CLA as well as trace amounts of other isomers (52). In 1997, only two companies (Natural ASA, Hovdebygda, Norway; PharmaNutrients Inc., Lake Bluff, IL) marketed CLA as a dietary supplement (62). Today numerous companies offer dietary supplements containing CLA; examples include Your Life®, Natrol® and Nature's Way® (63), Clarinol™, Met-Rx,

Dymatize® and EAS®, claiming a variety of health benefits. Supplement manufacturers recommend doses of 3-3.4 g per day for weight loss purpose (Clarinol, Tonalin) (64).

Physiological effects of *trans*-10, *cis*-12-CLA

CLA was discovered quite accidentally by Pariza and hargraves when they were investigating the carcinogenic properties of grilled beef; and they surprisingly and unexpectedly found that the fatty acids present in grilled beef exhibited anticarcinogenic rather than procarcinogenic properties (65). Ever since then, a large number of studies have been done using a 1:1 ratio of *cis*-9, *trans*-11-CLA and *trans*-10, *cis*-12-CLA both in vivo and in vitro (16), aiming to investigate CLA's several beneficial effects in health-related disorders. The results have shown that CLAs have anti-adipogenic (12, 13, 16), anti-carcinogenic (56, 66-72), anti-atherogenic (73-76), anti-diabetogenic (77, 78) and anti-inflammatory (79-82) properties. Among all the CLA isomers, the *cis*-9, *trans*-11-CLA and *trans*-10, *cis*-12-CLA isomers have various physiological functions and exhibit health benefits, individually or in association with each other (83, 84). Park et al. were the first to demonstrate that CLA mixture of 50% *cis*-9, *trans*-11-CLA and 50% *trans*-10, *cis*-12-CLA modulated body composition by decreasing body fat mass and increasing lean body mass (13). Experiments conducted later have confirmed that *trans*-10, *cis*-12-CLA is the isomer responsible for the changes in vivo and in vitro, particularly in body composition and lipid

metabolism (12, 14-16, 33, 85).

Trans-10, cis-12-CLA's effect on adipose tissue

In vitro studies

Brown and McIntosh found that *trans-10, cis-12-CLA* attenuated TG content and differentiation in primary cultures of human adipocyte (86). Similarly, a study demonstrated that *trans-10, cis-12-CLA* treatment at doses to those found in serum from rodents reduced adipogenesis and lipid droplet accumulation (87). In a study conducted by Park et al., *trans-10, cis-12-CLA* reduced TG content in the 3T3-L1 adipocytes (88).

In vivo studies

Dietary *trans-10, cis-12-CLA* supplementation has been demonstrated to be extremely effective at reducing the fat content ($\geq 60\%$) in a number of rodent models and pigs (89). Navarro et al. investigated the effect of 6 weeks of supplementation of 0.5% linoleic acid, *cis-9, trans-11-CLA* or *trans-10, cis-12-CLA* in atherogenic diet-fed hamsters. Although there was no difference in body weight, fat mass decreased significantly in *trans-10, cis-12-CLA*-fed hamsters (90). Another study showed that intake of *trans-10, cis-12-CLA* isomer for 3 weeks in genetically obese mice decreased gain in body weight and white fat pad weight (91). In a study conducted by Kang et al. using wild-type and

stearoyl-CoA desaturase 1 (Scd1) null mice, *trans*-10, *cis*-12-CLA decreased fat mass and enhanced mRNA expression of lipogenic enzymes, fatty acid synthase (Fasn) and uncoupling protein 2 (Ucp2), suggesting that antiobesity effects of *trans*-10, *cis*-12-CLA is independent of Scd1 gene expression and enzyme activity (92). House et al. conducted a study using a polygenic obese line of mice fed 1% *trans*-10, *cis*-12-CLA and showed that adipose mass was 30% less in the epididymal depot of CLA-fed mice, 27% less in the mesenteric depot and 58% less in brown adipose tissue after 14 days of CLA treatment (28). In contrast, a study in adult male Wistar rats fed with diets containing 1% *trans*-10, *cis*-12-CLA in combination with moderate physical activity for 6 weeks did not observe any effect on body composition or body weight (93). Based on the studies above, it is suggested that adult animals have not reproduced the dramatic results seen in growing animals (51). Additionally, mice are more sensitive to CLA than rats in losing fat mass (51), as Kim et al. stated that CLA-induced reductions in body fat gain varied in different species as following: mice > hamster > rats (94).

Some of the mechanisms suggested to be involved in fat reduction with *trans*-10, *cis*-12-CLA intake are increased energy expenditure (95), increased fat oxidation (96), decreased adipocyte size (97), and inhibition of enzymes involved in fatty acid metabolism and lipogenesis (83, 98, 99).

Clinical studies

Although the body fat-lowering effect of *trans*-10, *cis*-12-CLA has also been reported in humans, most clinical studies have not reflected the dramatic findings obtained in vitro and in vivo studies (51). In a controlled, randomized, double-blind parallel group study, the participants were given 2.2-4.2 g/d of *trans*-10, *cis*-12-CLA, or *cis*-9, *trans*-11-CLA, or combined for 4-12 wk (100). The results showed a certain reduction of the proportion of body fat especially abdominal fat by *trans*-10, *cis*-12-CLA (100). It should be noted that the loss of body fat is 40-50% greater in mice than observed in humans (61); however, mice are generally fed approximately 5 times more CLA than humans (per kilogram body weight) (101). In another randomized, double-blind controlled trial, 60 abdominally obese men were treated with 3.4 g/day CLA mixture, purified *trans*-10, *cis*-12-CLA or placebo for 12 weeks (46). The study found that *trans*-10, *cis*-12-CLA decreased body fat, sagittal abdominal diameter and weight versus baseline, but the difference was not significantly different from placebo; besides, the CLA mixture did not change body composition or weight compared with placebo (46).

Malpuech-Brugere et al. examined the effects of *cis*-9, *trans*-10-CLA and *trans*-10, *cis*-12-CLA isomers at two different intakes on body composition in 81 middle-aged overweight healthy humans (102). This was a bicentric, placebo-controlled, double-blind, randomized study. After 6-week consuming daily a dairy product containing 3

g of high oleic acid sunflower oil, volunteers were randomized to receive daily either 3 g of high oleic acid sunflower oil, 1.5 g of *cis*-9, *trans*-11-CLA, 3 g of *cis*-9, *trans*-11-CLA, 1.5 g of *trans*-10, *cis*-12-CLA, or 3 g of *trans*-10, *cis*-12-CLA administered as TG in a dairy product for 18 weeks. At the end of the 24 weeks, they did not find any significant differences in body composition and energetic uptake among the groups. Similarly, a study investigating the effects of 3 doses of highly enriched *cis*-9, *trans*-11 or *trans*-10, *cis*-12-CLA in healthy men did not demonstrate any significant alteration caused by any of those two isomers in the body composition of healthy adults (103).

In the vast majority of the studies with human volunteers, CLA mixtures rather than pure isomers have been used for supplementation (104). The effectiveness of CLA on humans is controversial. This may be partly because CLA dosage used in human studies is much lower than doses used in animal studies (51). For example, subjects in the human study received approximately 0.05 g/kg body weight CLA (105), the mice received 1.07 g/kg body weight CLA (106), which was 20 times the human dose based on body weight. Moreover, most animal studies have been in growing mice or rats, whereas studies in humans were mostly in mature volunteers whose changes in body composition in response to CLA supplementation have not been as dramatic as those seen in young animals (51).

Trans-10, cis-12-CLA's effect on liver

Although CLA seems to be a promising substance as regards reduction of body fat, it is important to consider the possibility of adverse effects found in animal models and more recently in specific groups of humans (49). Liver has been reported to be an important target for the effects of CLA on lipid metabolism (36). Many recent studies revealed that CLA could considerably reduced body fat mass but accompanied by hepatic steatosis (5, 12, 21-25). They also verified that *trans-10, cis-12-CLA* is the isomer responsible for the presence of fatty liver in mice associated with the loss of adipose tissue (12, 25, 31, 33, 107). The studies discussed in this section were summarized in Table A.5-A.7.

In vitro studies

A study conducted by Eder et al. showed that treating HepG2 cells with *trans-10, cis-12-CLA* increases the concentration of saturated fatty acid (SFA) in various cell lipid fractions at the expense of monounsaturated fatty acid (MUFA) (108), which confirms the investigation conducted by Choi et al. that clearly demonstrated that *trans-10, cis-12-CLA* strongly suppresses $\Delta 9$ -desaturase (109). Choi et al. found that treating HepG2 cells with *trans-10, cis-12-CLA* did not cause changes in the Scd gene transcription, mRNA and protein levels; however, this isomer decreased both the Scd activity as well as the levels of MUFAs, suggesting that in HepG2 cells the it regulates human Scd activity mainly by a

posttranslational mechanism (109). Since data from Belury and Kempa-Stedzko (110) suggested that liver microsomes may desaturate linoleate as well as CLA, Bretillon et al. (111) utilized male Wistar rat liver microsomes to investigate the effects of *trans*-10, *cis*-12-CLA on the desaturation of C18 fatty acids. They found that *trans*-10, *cis*-12-CLA significantly inhibited the conversion of stearic acid and the $\Delta 6$ desaturation of LA at the highest inhibitor/substrate (I/S) ratio. Another study demonstrated significant cytotoxic effects of 1 μ M *trans*-10, *cis*-12-CLA on dRLh-84 rat hepatoma cells (50).

In vivo studies

Studies in *trans*-10, *cis*-12-CLA supplementation have shown inconsistent results when comparing its effects on liver metabolism in different animal species. Thus, as Moya-Camarena and Belury suggested, there was the existence of a species-specific response of liver to CLA (and *trans*-10, *cis*-12-CLA) feeding (112).

Numerous studies have shown that feeding *trans*-10, *cis*-12-CLA leads to an increase in liver weight in mice. As mentioned previously, the study observing a reduction in adipose tissue in response to 1% *trans*-10, *cis*-12-CLA supplementation for 14 days, found that livers of the CLA-fed mice accumulated more fat and mass than the livers from LA-fed mice (control group) (32). Clément et al. showed that C57B1/6J female mice fed a diet enriched in *trans*-10, *cis*-12-CLA (0.4% w/w) for 4 weeks resulted in a dramatic

decrease in the mass of peri-uterine white adipose tissue (WAT) but accompanied by triggering a massive enlargement of the liver (3.1-fold increase) with typical features of a fatty liver: pale color and accumulation of intracellular lipids (33). They also suggest that *trans*-10, *cis*-12-CLA-induced hyperinsulinemia may trigger liver steatosis by inducing both fatty acid uptake and lipogenesis (33). Another group using aging female C57B1/6J mice pointed out that feeding 0.5% *trans*-10, *cis*-12-CLA for 6 months reduced the fat mass and induced liver hypertrophy accompanied by the accumulation of lipids in liver (48). In a study conducted by Degrace et al., C57BL/6j male mice were randomized to receive either 10g of oleic acid, *cis*-9, *trans*-11-CLA, or *trans*-10, *cis*-12-CLA for 4 weeks (30). The study observed a marked regression of the periepididymal adipose tissue (PAT) and a concomitant enlargement of liver associated with a severe fat accumulation (30). Their follow-up study confirmed their previous findings and found that the livers of *trans*-10, *cis*-12-CLA-fed mice had 170 μmol more TG and 1.1 μmol more total protein compared to those of controls (31). Due to the increased fatty acid oxidation capacities, they also suggested that hepatic steatosis was not caused by impaired fatty acid oxidation in C57BL/6J mice fed the *trans*-10, *cis*-12-CLA (31). A study using eighteen C57BL/6N female mice fed with either a control diet or diets supplemented with 0.5% *cis*-9, *trans*-11-CLA or 0.5% *trans*-10, *cis*-12-CLA for 8 weeks, found that the weights of the livers in the mice fed *trans*-10, *cis*-12-CLA was significantly ($p < 0.05$) greater than those in the control and *cis*-9, *trans*-11-CLA groups;

similarly, the weight of total liver lipids was approximately 4 times greater in the *trans*-10, *cis*-12-CLA than other groups, which was mainly due to an increase (5-fold) in the TG fractions, but cholesterol (3-fold), cholesterol esters (3-fold), and free fatty acids (2-fold) were also significantly increased (25). House et al. measured gene expression during *trans*-10, *cis*-12-CLA-induced fat reduction in polygenic obese line of mice. They observed an 33% increase ($p < 0.0001$) in liver weight by day 14 with 61% more fat present in the liver of *trans*-10, *cis*-12-CLA-fed mice compared with LA-fed mice ($p < 0.001$) (28). Similarly, Warren et al. also found that livers from the mice fed *trans*-10, *cis*-12-CLA contained 5 times more lipids than in the control group, with 30% lipids of the liver wet weights compared with 12% lipids of the liver wet weights in the control group (113). According to the studies (25, 30, 32, 33) that also measured the hepatic TG content, hepatic enlargement was associated with a 3- to 7-fold increase in TG content. Furthermore, our previous study suggested the lower threshold for these hepatic responses may be 0.15% *trans*-10, *cis*-12-CLA or less in a diet (24).

Several studies in hamsters have demonstrated different effects of *trans*-10, *cis*-12-CLA on liver compared with other rodent species, such as the mouse and the rat (114). Zabala et al. also observed a significantly greater liver weight ($p < 0.05$) produced by 5 g *trans*-10, *cis*-12-CLA, but with significantly lower TG content ($p < 0.01$) in 9-week-old male hamsters, indicating that the increased liver weight was not due to increased fat

accumulation (114). Moreover, they pointed out that the liver TG content in those mice was even significantly lower than that in the linoleic acid group; and, that reduced fat deposition in liver was not apparently due to changes in lipogenesis and should be ascribed to an increase in fatty acids oxidation (114). Another group showed that feeding male F1B hybrid hamsters with *trans*-10, *cis*-12-CLA (6.6 g/kg diet) for 8 weeks reduced weight gain as well as increased liver weight by 25% but not liver lipids (85).

In the study conducted by Gudbrandsen et al., feeding male Zucker fa/fa rats diets supplemented with 1% *trans*-10, *cis*-12-CLA for 10 days reduced the liver TG content without improving the overall adiposity or any change in liver weight (36). On contrary, another study observed a 33% increase in liver lipid content in the growing female Zucker fa/fa rats fed *trans*-10, *cis*-12-CLA (1.5 g CLA/kg BW) for 21 days (89).

Clinical studies

So far CLA supplement on the market has been a mixture of *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA at an equal proportion and supplement manufacturers recommend doses of 3-3.4 g per day for weight loss purpose (Clarinol, Tonalin) (64). Most clinical trials studying the safety of CLA in humans typically lasted between 4 and 13 weeks and dosages were 1.4-4.2 g, with a few exceptions that employed 6-6.8 g (115). A very recent study (64) determining the safety of a very high-dose (14.6 g *cis*-9, *trans*-11-CLA and 4.7 g *trans*-10,

cis-12-CLA per day) of CLA which was almost 3 times higher than the highest dose in previous studies on CLA (115), did not find any clinically relevant effects on liver function tests of the 19 participants after taking CLA supplement for 3 weeks. Thus, it ended up with the conclusion that doses of up to 3 g per day as commonly ingested from CLA supplements are unlikely to affect liver function at least over a period of weeks (64). However, the results of this study did not suggest that *trans*-10, *cis*-12-CLA would not cause any liver damage in such high dose. First, the content of *trans*-10, *cis*-12-CLA in CLA mixture they used was much lower than the other isomer. Because these two CLA isomers are responsible for different biological functions, so the high *cis*-9, *trans*-11-CLA content might affect or mask the influence of *trans*-10, *cis*-12-CLA on liver. Second, the subjects were mostly young women and only 19 participants were involved in this study; thereby, they were not very representative. Finally, the study only measured several biomarkers of liver function and those measurements could not indicate any change in liver weight.

A randomized, crossover, single-blind clinical trial (116) was carried out by Venkatramanan et al. in 11 moderately overweight, borderline hyperlipidemic individuals who consumed milk enriched with a 4.2% synthetic mixture of *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA isomers providing 1.3 g/d of CLA. The results indicated that supplementation with CLA-enriched milk did not significantly affect liver function or body weight (116). Since *trans*-10, *cis*-12-CLA is responsible for reducing fat mass and

increasing liver weight, the relatively low amount of this isomer used in the study may be at a level below the threshold necessary to elicit changes in body composition (116). However, this study utilized CLA in a food format with milk, which could possibly enhance the absorption and metabolism of the CLA supplement compared with those in a purified compound format. Similarly, neither Berven et al. (117) nor Blankson et al. (45) reported any clinically important changes in the blood levels of liver enzymes during the 12-week treatment with a mixture of *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA at an equal proportion. Lowery et al. also found no effect of the CLA mixture on the liver enzyme transferases in novice body builders supplemented with a two-isomer preparation (3.9 g/d) for 6 weeks (118).

In a study (119) trying to determine the safety of CLA in Japanese, 60 healthy overweight male were randomly assigned to three groups: 5.4 g CLA-triacylglycerol (3.4 g CLA), 10.8 g CLA- triacylglycerol (6.8 g CLA) and placebo daily for 12 weeks. The CLA-triacylglycerol contained *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA at an equal proportion (119). The study observed a slight increase in the level of liver enzymes (serum AST and ALT activities) in the 6.8 CLA group at 12 weeks; however, these changes were within the normal range (119). The results also indicate that CLA at a dose of 3.4 g/d is a safe dietary level in healthy Japanese populations based on no clinically significant changes in vital signs.

The long-term safety of CLA supplement was evaluated by a randomized, double-blind study consisting of 50 obese humans for one-year period (120). Subjects were randomized to receive either 6 g/day of CLA mixed isomers (as 7.5 g/day Clarinol™) containing 37.3% *cis*-9, *trans*-11-CLA and 37.6% *trans*-10, *cis*-12-CLA, or placebo (high oleic sunflower oil) (120). The results showed that body composition did not differ between groups and liver function was not affected, in terms of ALT, AST and alkaline phosphatase in serum (120). The study concluded that CLA as Clarinol™ is safe for use in obese humans for at least one year (120).

So far there have been no clinical studies investigating the effects or safety of *trans*-10, *cis*-12-CLA supplementation by utilizing the purified isomer. Hence, we can get few hints from those studies using mixed isomers which may negate one another. Moreover, doses used in human trials were much lower than those used in animal studies (86). Therefore, in future, a large number of studies using different amounts of purified *trans*-10, *cis*-12-CLA supplement need to be carried out to investigate its effect on liver weight in humans. Furthermore, those studies need to be duplicated in other labs giving emphasis to men and women, age groups, ethnic background or food style (121). All in all, *trans*-10, *cis*-12-CLA-specific dose-titrated world-wide network clinical trials combined with mechanistic studies in cultures of primary cells should provide the much needed insight on potential human applications for this isomer (121). When extrapolating rodent data, such as

mouse, rat and hamster, to the human situation, the differences in species' sensitivities are of great importance (122, 123).

Hypothesized working mechanisms of *trans*-10, *cis*-12-CLA in liver

Increased hepatic lipogenesis

It was suggested that fatty liver could be a consequence of the increased lipogenesis in the liver in compensating for the reduction of fat deposition in the adipose tissue (124-126). A study found that increased fatty acid synthesis seemed to contribute to the *trans*-10, *cis*-12-CLA-induced fatty liver, evidenced by the increased expression of four lipogenic genes by more than 2-fold, including acetyl-CoA carboxylase (Acaca), fatty acid synthase (Fasn), malic enzyme (Me) and stearyl CoA desaturase-1 (Scd1) (127). Indeed, the strong and specific induction of genes expressed at only very low levels in the normal liver, such as those encoding PPAR γ , Fat/Cd36, and Albp, was observed in mice fed the *trans*-10, *cis*-12-CLA (33). These modifications may be accounted for by the concomitant induction of Srebf1 gene (33), which is known to be a major regulator of hepatic lipogenic program (128). Jaudszus et al. in a very recent study speculated that the reduction in the white adipose tissue in the *trans*-10, *cis*-12-CLA group was likely compensated by the hepatomegaly due to redistribution, altered oxidation and/or increased de novo synthesis of fatty acids (5). Therefore, the profound hepatic steatosis showed by animals consuming

trans-10, *cis*-12-CLA isomer has been attributed to increased hepatic TG, cholesterol, cholesterol esters and free fatty acids (FFAs) (25), reflecting increased hepatic fatty acids synthesis (124). These observations strongly indicated that an increase in lipogenesis is primarily responsible for the *trans*-10, *cis*-12-CLA-dependent accumulation of TG in the liver (129).

On contrary, a study (114) assessing the effects of *trans*-10, *cis*-12-CLA on mRNA levels and the activities of several lipogenic enzymes in hamsters, demonstrated that *trans*-10, *cis*-12-CLA isomer produced significantly greater liver weight but also significantly decreased liver fat accumulation. The study also observed no changes in mRNA levels or the activities of lipogenic enzymes (114). In terms of HepG2 cells, treatment of *trans*-10, *cis*-12-CLA did not cause changes in the Scd gene transcription, mRNA and protein levels (109).

Direct activation of PPARs

It was suggested that the observed *trans*-10, *cis*-12-CLA-mediated increased liver weight result from the direct activation of peroxisome proliferator-activated receptors (PPARs) (121, 130) and subsequent “switching on and/or off” of the target genes to elicit a host of biochemical pathways (131). According to the proposed CLA-mediated signal transduction by Benjamin et al. in Figure 2.2, during gene regulation, the well characterized

PPARs (α , β or γ subtypes) bind to the peroxisome proliferator responsive element (PPRE) on the nuclear DNA as heterodimers with the α , β or γ subtypes of the retinoic acid receptor (RXR), which in turn, needs to be activated by *cis*-9-retinoic acid to effect target gene transcription (132, 133).

As *trans*-10, *cis*-12-CLA has been reported to be a potent ligand for Ppar α (134), the underlying regulations might imply the activation of Ppar α , which was shown to repress apoptosis (135) and therefore to favor liver enlargement. Rasooly et al. observed a 65% reduction in Ppar α gene expression in the liver of the mice fed *trans*-10, *cis*-12-CLA diet (127). The down-regulation of Ppar α in the liver seems to be pivotal for the hepatic abnormalities (113). LaRosa et al. pointed out that suppression of Ppar α is considered to be an integrated response to cellular stress, e.g. due to lipid peroxidation, leading to the activation of the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF κ B) (136). Furthermore, down-regulation of Ppar α as a central regulator of lipid homeostasis determines the inhibition of β oxidation and results, for example, in an over-expression of Srebf1 in the liver (137). Srebf1 controls a multitude of genes involved in hepatic de novo lipogenesis, such as Dgat (30) and Scd (5). See Figure 2.3.

Indirect mechanism: hyperinsulinemia

Several lines of evidence strongly suggest that the liver steatosis occurring in

trans-10, *cis*-12-CLA-fed mice is secondary to hyperinsulinemia, which causes high levels of fatty acid uptake and synthesis (33). First, because white adipose tissue plays a crucial role in metabolizing and converting glucose to fatty acid for storage purposes, the large decrease of fat pad mass in response to dietary *trans*-10, *cis*-12-CLA may result in a retardation of whole-body glucose metabolism (129). Therefore, Ide et al. speculated that there is a possibility that the counteraction of *trans*-10, *cis*-12-CLA-mediated induction of hepatic lipogenesis aggravates glucose intolerance and hyperinsulinemia, despite being potentially effective in preventing fatty liver (129). A large increase in lipogenesis and accumulation of TG in the liver after *trans*-10, *cis*-12-CLA treatment may represent the physiological response of the animal to metabolize excess glucose to fatty acid and store it as TG in the liver rather than in adipose tissue (129). Second, Clément et al. pointed out that fatty liver was not observed in *cis*-9, *trans*-11-CLA-fed mice, which remained normoinsulinemic (33). Third, hyperinsulinemia is associated with the induction of Ppar γ gene expression in the liver and with liver steatosis in several mouse models (126). Fourth, insulin is known to upregulate Ppar γ gene expression in adipocytes (138) and to induce Fas gene expression in the liver (139, 140). Finally, Clément et al. also demonstrated down-regulation of Pck1, strongly suggesting that the livers of *trans*-10, *cis*-12-CLA-fed mice remain sensitive to insulin (33).

Several in vivo studies have reported that dietary CLA caused marked glucose

intolerance and hyperinsulinemia (33, 141). Clément et al. showed that the mice fed a diet enriched in *trans*-10, *cis*-12-CLA (0.4% w/w) for 4 weeks developed lipodystrophy, hyperinsulinemia and fatty liver (33). In their study, a dramatic increase in non fasting plasma insulin levels was observed in mice fed the *trans*-10, *cis*-12-CLA-enriched diet, these mice displaying insulin concentrations 10 times higher than mice fed control and LA- or *cis*-9, *trans*-11-CLA-enriched diets; and, despite this marked hyperinsulinemia, plasma glucose concentration remained within the normal range in these mice (33). Another study found that *trans*-10, *cis*-12-CLA diet promoted insulin resistance and increased serum glucose ($p = 0.025$) and insulin ($p = 0.01$) concentrations in ob/ob C57BL-6 mice (141). Risérus et al. (142) conducted a clinical study including 57 non-diabetic abdominally obese men to randomly receive either 3.4 g *trans*-10, *cis*-12-CLA or control oil. After 12-week treatment, they demonstrated that *trans*-10, *cis*-12-CLA induced hyperproinsulinaemia that was related to impaired insulin sensitivity, independently of changes in insulin concentrations in obese men (142). Thus, they suspected the use of weight-loss supplements containing this CLA isomer (142).

However, the cause of the dramatic hyperinsulinemia triggered by the *trans*-10, *cis*-12-CLA remains to be determined.

Insufficient hepatic VLDL secretion in liver

In general, TG which is formed by esterification of fatty acid derived from plasma, is stored as droplets or secreted in the form of VLDL whose secretion depends on the synthesis of TG as well as apoB-containing lipoproteins which are major lipoproteins in transporting the hepatic lipids out of the liver (143). Thus, an alteration of VLDL secretion rates could also result in liver fat accumulation (30).

A study (144) aimed to investigate the effects of *trans*-10, *cis*-12-CLA on liver lipid metabolism in cultured HepG2 cells, which are human hepatoma cells and are known to exert a number of human liver functions, including fatty acid uptake, lipid synthesis, VLDL assembly and VLDL secretion (145). A novel finding in this study is that this CLA isomer suppressed TG secretion in spite of the fact that it increased 4-fold the cellular TG content (144). Since the secreted TG is mainly contained in VLDL, the decreased TG secretion by *trans*-10, *cis*-12-CLA reflects a diminished secretion of VLDL (144). Yotsumoto et al. found that apoB secretion by HepG2 cells treated with 10 μ M *trans*-10, *cis*-12-CLA for 24 hours was decreased markedly when compared with the control group; and this CLA isomer also inhibited cellular TG and cholesteryl ester synthesis (146). Thus, this study suggests that *trans*-10, *cis*-12-CLA inhibited TG synthesis and reduced apoB secretion (146).

In terms of in vivo studies, Degrace et al. (30) demonstrated that the steatosis

accompanying the fat loss induced by *trans*-10, *cis*-12-CLA in C57BL/6j mice was not due to an alteration of the liver lipoprotein production that was even increased. The data obtained in that study showed that the ability of the total liver to secrete VLDL was practically two times greater in *trans*-10, *cis*-12-CLA-fed mice than in controls (30). Therefore, they indicated that the liver TG accumulation could not be attributed to a decreased VLDL secretion and that the VLDL overproduction from the enlarged liver was however insufficient to eliminate the flux of fatty acid entering the whole esterification and lipoprotein assembling/secretion pathway (30). According to the different results between HepG2 cells and mice, the explanations regarding the relations between the setup of the liver steatosis and VLDL secretion in mice fed diets containing purified *trans*-10, *cis*-12-CLA are very scarce and still confused.

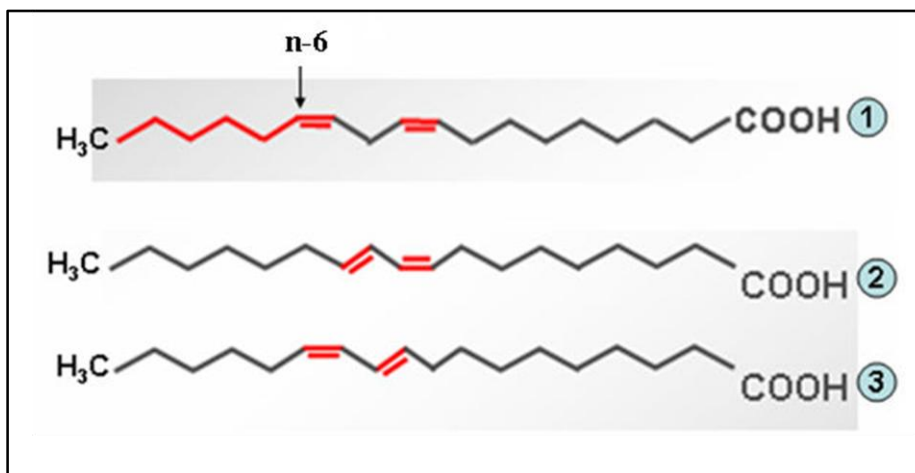


Figure 2.1 Structure of LA and its major CLA derivatives. 1. LA; 2. *cis*-9, *trans*-11-CLA; 3. *trans*-10, *cis*-12-CLA (121).

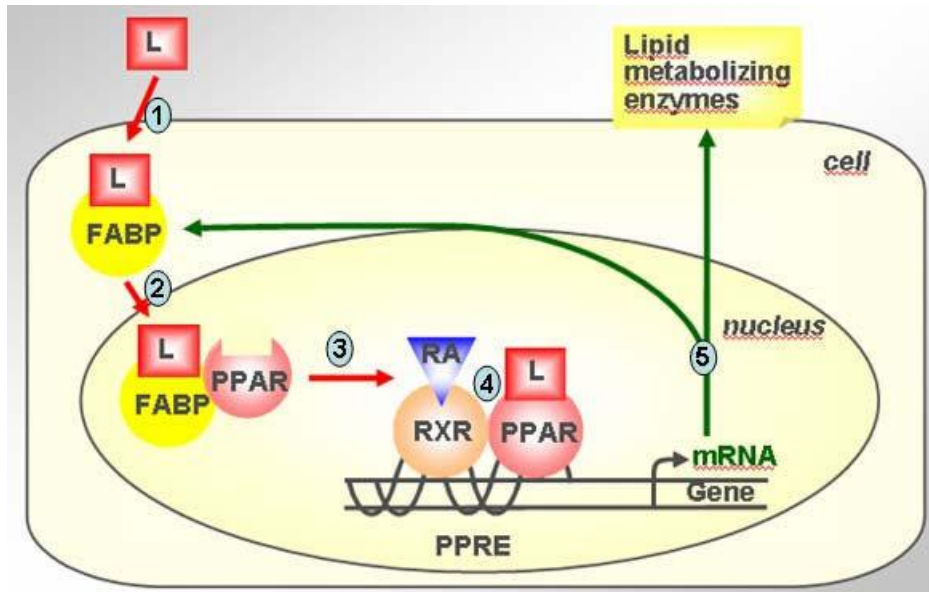


Figure 2.2 Proposed CLA-mediated signal transduction. The dietary lipid nutrient L (L = here CLA) crossing the cell membrane with the help of specific membrane-bound fatty acid transporters and binds to tissue-specific fatty acid binding protein (FABP) in the cytosol; 2. The L/FABP complex enters the nucleoplasm, where L is transferred to the specific peroxisome proliferators activated receptor (PPAR) subtype; 3. The L/PPAR complex heterodimerises with retinoic acid (RA)/retinoic acid receptor (RXR) subtype; 4. This heterodimer binds to the peroxisome proliferator responsive element (PPRE) on the target gene; and 5. Specific gene expression occurs, whose products act intra- or extracellularly to elicit a host of various biological functions. (121)

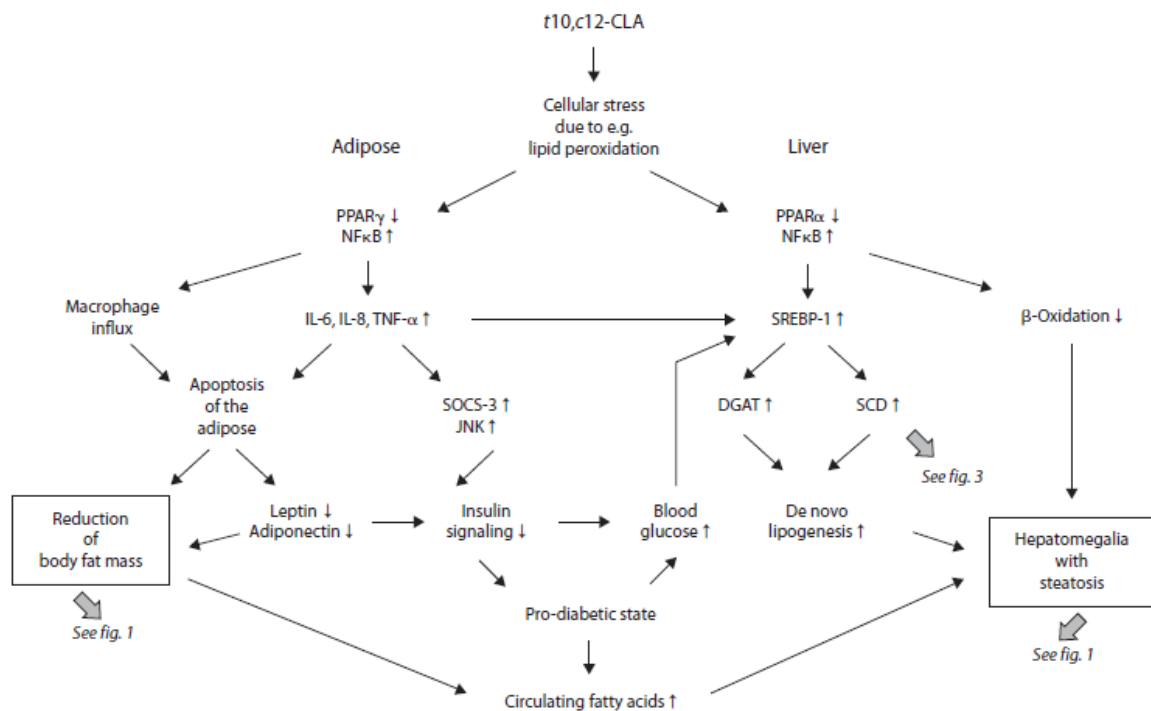


Figure 2.3 Observations from the present study in relation to previously suggested and approved mechanisms underlying the *trans*-10, *cis*-12-CLA-induced lipodystrophic alterations in rodents (5).

CHAPTER 3

Methodology

Animals, diets, and sampling

Male CD-1 mice from Harlan (Madison, WI), aged 3- to 4-week-old, were divided into two experimental groups of ten mice each. The mice were housed individually with access to food and water at all times. A 12-h light/12-h dark cycle was maintained throughout the study. For 7 d before the start of the study, all mice were fed Harlan Teklad (Harlan, Madison, WI) Global Rodent Diet (2018) (18% protein, 5% fat) with 3% (w/w) high-oleic sunflower oil added. On day 1 of the study, mice were randomly assigned to receive diets containing 3.0% high-oleic sunflower (control) or 2.70% high-oleic sunflower oil + 0.30% *trans*-10, *cis*-12-CLA. The free FA form of *trans*-10, *cis*-12-CLA (>95% purity) was obtained from Natural Lipids (Hovdebygda, Norway). Mice were fed daily at 16.00 hours, and food refusals were weighed to estimate intake during the previous 24 h. Samples of each diet were obtained each week and stored at 4°C prior to FA analysis. Table 3.1 gives an overview of the FA composition of the diets fed to growing male mice. Data on body weight, feed intake, and tissue fatty acid profiles have been published previously (24).

At the end of weeks 2 and 6, five mice per dietary treatment were anesthetized with Metofane ® (Pitman-Moore, Inc, Washington Crossing, NJ) prior to cervical dislocation. The liver was removed, rinsed with diethylpyrocarbonate (Sigma, St. Louis, MO)

in distilled water (1:1000, v/v), and weighed. A portion of liver was frozen in liquid nitrogen and stored at -80°C prior to RNA extraction and gene expression analysis. The remainder of the liver was stored at -20°C prior to FA analysis, which is reported in the previous study (24).

Extraction and analysis of RNA and semiquantification by RT-PCR

Liver RNA was extracted using TRI REAGENT[®] (MRC, Cincinnati, OH) and according to the manufacturer's instructions. Genomic DNA was removed from RNA with DNase (Qiagen) using RNeasy Mini Kit columns (Qiagen, Germany). RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The purity of RNA (A_{260}/A_{280}) for all samples was above 1.8. RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies). A portion of assessed RNA was diluted to 100 ng/μl using DNase-RNase free water prior to reverse transcriptase.

Each cDNA was synthesized by RT-PCR using 100 ng RNA, 1 μl dT18 (Operon Biotechnologies, AL), 1 μl 10 mmol/L dNTP mix (Invitrogen Corp., CA), 1 μl random primers (Invitrogen Corp., CA), and 10 μl DNase/RNase free water. This mixture was incubated at 65°C for 5 min and kept on ice for 3 min. A total of 6 μl of Master Mix composed of 4.5 μl 5X First-Strand Buffer, 1 μl 0.1 M DTT, 0.25 μl (50u) of SuperScript[™] III RT (Invitrogen Corp., CA), and 0.25 μl of RNase Inhibitor (10U, Promega, WI) was

added. The reaction was performed in an Eppendorf Mastercycler® Gradient using the following temperature program: 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min.

cDNA was diluted 1:4 with DNase/RNase free water.

Quantitative PCR (qPCR) was performed using 4 µl of diluted cDNA combined with 6 µl of a mixture composed of 5 µl 1×SYBR Green master mix (Applied Biosystems, CA), 0.4 µl each of 10 µM forward and reverse primers, and 0.2 µl DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems, CA). Each sample was run in triplicate and a 5 point relative standard curve plus the non-template control (NTC) were used (User Bulletin #2, Applied Biosystems, CA). The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems, CA) using the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C (denaturation), and 1 min at 60°C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95°C for 15 s plus 65°C for 15 s. Complete details regarding amplification of target genes can be found at <http://docs.appliedbiosystems.com/pebi docs/04364014.pdf>.

Selection and evaluation of internal control genes (ICG)

Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems) using a five-point standard curve, as required for the

evaluation of gene stability. Stability (M = gene – stability measure) using geNorm (147) refers to the constancy of the expression ratio between two non-co-regulated genes among all samples tested. The more stable the expression ratio among two genes, the more likely that the genes are appropriate internal controls, i.e. two ideal control genes should have an identical expression ratio in all samples regardless of experimental conditions, cell, and/or tissue type. The lower the M value, the higher the stability. geNorm also performs an analysis to determine the utility of including more than 2 genes for normalization by calculating the pairwise variation (V) between the normalization factor (NF) obtained using n genes (best references) (NF_n) and the NF obtained using $n+1$ genes (addition of an extra less stable reference gene) (NF_{n+1}). A large decrease in the pairwise variation indicates that addition of the subsequent more stable gene (i.e. with lowest M value) has a significant effect and should be included for calculation of the NF (147). Once the most stable internal reference genes are selected, the NF is calculated using the geometrical average between them to normalize qPCR data.

At the beginning, a total of seven genes were selected as internal control genes, including β -actin, β_2 microglobulin (β -2M), Procollagen-lysine-2-oxoglutarate 5-dioxygenase 3 (PLOD3), neuroglobin (NGB), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sentrin specific peptidase 7 (SENP7), and syntaxin 8 (STX8). According to the results generated by geNorm, β -actin, β_2 microglobulin (β -2M),

Procollagen-lysine-2-oxoglutarate 5-dioxygenase 3 (PLOD3), and neuroglobin (NGB)

expression data were the four most stable genes among the ones tested as internal controls.

Selection of genes and primer design

Thirty-eight genes encompassing networks with central biological functions in lipid metabolism were evaluated (Figure 1.1). Those genes encode proteins required for fatty acid uptake, fatty acid oxidation, de novo fatty acid synthesis, lipolysis, lipogenesis, ketogenesis, carbohydrate metabolism, apoptosis, inflammation, stress response, growth factor signaling, transcriptional regulation of lipogenesis and lipid droplet formation.

Primers were designed with Primer Express software fixing the amplicon length to 100-120 bp with low specific binding at the 3'-end (limit 3'-G+C; Applied Biosystems). Primers for the thirty-eight selected genes and the four internal control genes are shown in Table S2.

Primers were aligned against publicly available databases using BLAST software at the National Center of Biotechnology Information and also UCSC's Mouse Genome Browser Gateway

(<http://genome.ucsc.edu/cgi-bin/hgGateway?hgsid=183612843&clade=mammal&org=Mouse&db=0>). Prior to qPCR, primers were tested by semiquantitative PCR using a total of 20 µl mixture composed of 8 µl pooled cDNA, 10 µl SYBR Green, 1 µl each of forward and reverse primers. The PCR reaction was set to 2 min at 50°C, 10 min at 95°C, 40 cycles of 15

s at 95°C (denaturation). PCR products were run in a 3% agarose gel (Invitrogen) stained with ethidium bromide. PCR products were sequenced at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana-Champaign. Aliquots of all PCR products are stored to enable additional confirmatory sequencing of PCR products in the future if required.

Gene network analysis

Gene networks were evaluated using Ingenuity Pathway Analysis[®] (IPA; <http://www.ingenuity.com>), Redwood City, CA). This is a web-based application that enables the discovery, visualization, and exploration of interaction networks. The software relies on currently known relationships (i.e., published manuscripts) among human, mouse and rat genes/proteins.

Statistical analysis

Data were analyzed as a completely randomized design with a factorial arrangement of treatments using the MIXED procedure of SAS[®] (Windows version 9.1, Cary, NC). Interaction was dietary treatment \times week. Leastsquare means \pm SEM (combined observations for weeks 2 and 6) are presented in tables and figures.

Relative mRNA abundance among transcripts

Efficiency of PCR amplification for each gene was calculated using the standard curve method ($E = 10^{-1/\log \text{ curve slope}}$). Relative mRNA abundance among measured genes was calculated as reported by Bionaz et al. (148), using the inverse of PCR efficiency raised to ΔC_t (gene abundance = $1/E^{\Delta C_t}$, where $\Delta C_t = C_t \text{ sample} - \text{geometric mean } C_t \text{ of 4 internal control genes}$). Overall mRNA abundance for each gene among all samples measured was calculated using the median ΔC_t . Use of this technique for estimating relative mRNA abundance among genes was necessary because relative mRNA quantification was performed using a standard curve (made from a mixture of RNA from several mouse tissues, which precluded a direct comparison among genes. Together, use of C_t values corrected for the efficiency of amplification plus internal control genes as baseline overcome this limitation. Description of genes measured and overall % relative mRNA abundance are reported in Table 1.1.

Table 3.1 Fatty Acid Composition of Diets Fed to Growing Male Mice for 6 Wk

Fatty Acid	<i>trans</i> -10, <i>cis</i> -12-CLA	
	0%	0.30%
10:0	0.02	0.02
12:0	0.04	0.05
14:0	0.12	0.12
16:0	9.26	9.18
<i>cis</i> -9-16:1	0.18	0.19
18:0	2.49	2.52
<i>trans</i> -11-18:1	0.02	0.02
<i>cis</i> -9-18:1	34.41	31.77
18:2n-6	47.34	46.28
<i>cis</i> -9, <i>trans</i> -11-18:2	0.02	0.32
<i>trans</i> -10, <i>cis</i> -12-18:2	0.00	2.67
18:3n-3	3.72	3.77
20:0	0.39	0.38
20:4n-6	0.02	0.03
Total (µg/mg of diet)	44.07	46.14

CHAPTER 4

Results and Discussion

Data from this study published previously (24) observed a reduced deposition of lipids in peripheral tissues as well as a 15-18% increase in liver weight of male mice in response to relatively low amounts of *trans*-10, *cis*-12-CLA in a dose-dependent manner; and, the increased liver weight was accompanied by an increase in hepatic total fatty acid content. Apart from our previous study, many other studies have shown that *trans*-10, *cis*-12-CLA increases liver weight and reduces adipose mass; and, those studies that also measured the hepatic TG content, hepatic enlargement was associated with a 3- to 7-fold increase in TG content as summarized in Table 4.2. In the present study, we examined a total of thirty-eight genes related to lipid metabolism using real-time PCR in the livers of mice fed *trans*-10, *cis*-12-CLA, aiming to explore the underlying cellular and molecular mechanisms causing increased liver weight and potentially impaired function. The results of hepatic gene expression in response to *trans*-10, *cis*-12-CLA were listed in Table 4.3-4.5.

***Trans*-10, *cis*-12-CLA increased fatty acid uptake in the liver**

Accumulating evidence suggests that an increase in lipogenesis and esterification due to dietary *trans*-10, *cis*-12-CLA is primarily responsible for liver triacylglycerol (TG) accumulation (33, 124, 129). Liver TG accumulation also could be a result of increased

hepatic uptake of circulating non-esterified fatty acids (NEFA) (149). In a study conducted by Gruffat et al., liver tissue samples of rats were incubated with 0.75 mM of fatty acid mixture (representative of circulating NEFA) and with 55 μ M *trans*-10, *cis*-12-CLA or oleate. They found that the uptake of CLA by hepatocytes was three times higher ($p < 0.01$) than for oleate (150). Passive diffusion of fatty acid across membranes plays a minor role compared with protein-mediated fatty acid uptake and the flip-flop mechanism (151). The main proteins involved in fatty acid uptake in non-ruminant cells include fatty acid translocase Fat/Cd36 (Cd36) (152) and fatty acid transport proteins (Slc27a) (153). Slc27a2 is a member of the family of fatty acid transport proteins (FATPs) (151) and is predominantly expressed in liver and kidney (153). Serum NEFAs traverse into the hepatocytes mainly by interaction of the NEFAs directly with Slc27a2 or by a preceding binding to cell-surface proteins, such as Cd36, which subsequently hands the NEFAs to Slc27a2 (151).

In the present study, Cd36 mRNA abundance accounted for 0.0005% whereas Slc27a2 accounted for 3.63% of total genes measured. However, Cd36 mRNA expression (CLA = 1.11, control = -1.47; $p = 0.001$) was greater with *trans*-10, *cis*-12-CLA treatment and there was no change for Slc27a2 expression (CLA = -0.04, control = -0.23; $p = 0.42$). The increased Cd36 expression suggests that *trans*-10, *cis*-12-CLA caused more NEFAs uptake into hepatocytes, which was in agreement with a higher hepatic FFA content

(two-fold) (25) as well as a lower serum FFA concentration in mice fed *trans*-10, *cis*-12-CLA (31). Furthermore, a previous study showed not only a significant increase in hepatic Cd36 expression in mice fed *trans*-10, *cis*-12-CLA, but also significant association between Cd36 expression and the degree of hepatic steatosis (154). Therefore, upregulation of Cd36, although not the most abundant NEFA transporter, is part of the mechanism leading to increased hepatic fatty acid uptake and hepatic steatosis induced by *trans*-10, *cis*-12-CLA.

***Trans*-10, *cis*-12-CLA stimulates lipogenesis and TG synthesis in liver**

Acaca and de novo fatty acid synthesis

Several authors suggested that de novo fatty acid synthesis may play a role in the onset of hepatic steatosis related to CLA supplementation (124, 129). Acetyl-CoA carboxylase (Acc) is one of the two enzymes required for long-chain fatty acid (LCFA) synthesis; the other is Fasn (155). ACC catalyzes the carboxylation of acetyl-CoA to generate malonyl-CoA, which is the committed step in this pathway (155). Two forms of Acc have now been identified: Acc- α (Acaca) and Acc- β (155). Acaca is involved in the synthesis of LCFAs, while Acc- β may be involved in the regulation of mitochondrial oxidation of fatty acids (156).

In our study, mRNA abundance of this gene accounted for 0.52% of total genes

measured. We observed that hepatic *Acaca* expression was significantly up-regulated (CLA = 0.72, control = 0.22; $p = 0.0027$) in response to *trans*-10, *cis*-12-CLA, suggesting that the liver was generating more malonyl-CoA which was then used for palmitic acid synthesis by the multifunctional enzyme *Fasn* (155). Results from Ashwell et al. (32) and Kelley et al. (25) support this hypothesis by observing more C16:0 in liver tissue of obese mice fed *trans*-10, *cis*-12-CLA than those fed a LA or corn oil diet.

However, there are some studies showing no significant changes in hepatic *Acaca* expression in mice fed *trans*-10, *cis*-12-CLA. In a study conducted by Ashwell et al., feeding mice 1% *trans*-10, *cis*-12-CLA for 14 days resulted in accumulation of 62.5% more fat in livers and a 33% increase in liver weight (32). They surprisingly found *Acaca* and other genes involved in fatty acid and glycerol lipid synthesis and/or degradation were not impacted by *trans*-10, *cis*-12-CLA (32). Zabala et al. also pointed out that *trans*-10, *cis*-12-CLA (5g/kg diet for 6 weeks) increased liver weight but reduced hepatic TG content without affecting mRNA levels and the activities of several lipogenic enzymes, including *Acaca*, in hamsters (114). In that study, the increased liver weight might be due to a significant increase in hepatocytes number (23% $p < 0.01$) instead of excessive fat accumulation (MT Macarulla, A Fernández-Quintela, A Zabala, V Navarro, E Echevarria, I Charruca, VM Rodríguez and MP Portillo, unpublished results). These findings suggest the existence of a species-specific response of liver to CLA feeding (112).

Moreover, although our study observed an upregulation of *Acaca* in mice fed *trans*-10, *cis*-12-CLA, increasing fate of fatty acid synthesis by stimulating *Acaca* may not guarantee that the fatty acids will be diverted towards TG biosynthesis (157), because the fatty acids may be used either for biosynthesis of TG or for degradation via β oxidation in the mitochondrial matrix (157).

Scd and TG synthesis

Stearoyl-CoA desaturase (*Scd*) is the rate-limiting enzyme involved in the synthesis of mono unsaturated fatty acids (16:1 or 18:1) from saturated fatty acids (16:0 or 18:0) (158). Although not in the G3P pathway of TG synthesis, *Scd* plays an important role in the synthesis of TG (158). A proper ratio of saturated to monounsaturated fatty acids contributes to membrane fluidity and alterations have been implicated in a variety of disease states (159). The expression of the mouse *Scd* gene is regulated by PUFAs and cholesterol at the levels of transcription and mRNA stability (160). Many studies have showed that *Scd* mRNA expression in adipose tissue decreased as the *trans*-10, *cis*-12-CLA content of the diet increased (5, 24).

We observed a significant upregulation of expression of *Scd* (CLA = 0.05, control = -0.92; $p = 0.0007$) in the liver of the mice fed *trans*-10, *cis*-12-CLA. mRNA abundance of *Scd* accounted for 2.53% of total genes measured. Guillen et al. supported our finding by

observing an increase in hepatic Scd expression in mice fed *trans*-10, *cis*-12-CLA (154). Moreover, they indicated that Scd had a significant association with the degree of hepatic steatosis (154). Because a large number of studies have shown that *trans*-10, *cis*-12-CLA reduces fat mass considerably, the upregulation of hepatic Scd mRNA due to feeding *trans*-10, *cis*-12-CLA may be a response to greater influx of 16:0 and 18:0 derived from adipose tissue. Moreover, the increased in de novo fatty acid synthesis via up-regulation of hepatic Acaca expression also may have contributed indirectly to the greater hepatic Scd expression. Together with the up-regulation of Gpam expression in the liver (discussed as below), *trans*-10, *cis*-12-CLA-induced hepatic steatosis may, at least in part, be attributed to stimulated lipogenesis and esterification. Our result was in agreement with the findings from previous studies (5, 32) which measured the ratio of saturated to monounsaturated fatty acids as an indicator of the enzymatic activity of Scd.

GPAM and TG synthesis

In the glycerol phosphate pathway to synthesize TG, acyl-chains from acyl-CoA are transferred consecutively to G3P produced either via glyceroneogenesis or through phosphorylation of glycerol released from adipose tissue during lipolysis (161). This reaction is catalyzed by G3P acyltransferase (Gpam) which resides in the outer mitochondrial membrane (162), and it is the first committed step in the synthesis of TG

(162, 163) via the glycerol phosphate pathway. Besides, Gpm is an enzyme that can switch the fate of fatty acids from β oxidation to glycerolipid synthesis (164, 165).

In our study, Gpm mRNA abundance accounted for 3.18% of total transcripts measured. Although a previous study found that *trans*-10, *cis*-12-CLA did not change statistically the hepatic expression of Gpm in mice (32), we observed a marked increase in hepatic Gpm expression (CLA = 0.44, control = -0.31; $p = 0.003$) in mice fed *trans*-10, *cis*-12-CLA, which was suggestive of stimulation of esterification in order to synthesize TG in liver. It could be possible that the concerted upregulation of both *Acaca* and Gpm was a mechanistic response to promote biosynthesis of TG, e.g. the up-regulation of *Acaca* stimulated *de novo* fatty acid synthesis, thus, generated more precursor for TG synthesis and resulting in increased Gpm expression.

Moreover, there is a potential that blood NEFAs derived and exported from adipose tissue went into liver and most of them would be esterified into TG via Gpm. From this point, synthesis of TG under catabolic conditions (i.e. as it would occur during lipolytic stimulation) is not a result of lipogenesis but esterification of blood derived NEFA and lower rates of oxidation of fatty acids to ketones and carbon dioxide. All in all, our results supported the hypothesis that *trans*-10, *cis*-12-CLA-induced lipogenesis and esterification are primarily responsible for liver TG accumulation (33, 124, 129).

Dgat1, Dgat2 and TG synthesis

There are two major biochemical pathways for TG synthesis: the monoacylglycerol (MAG) pathway and the G3P pathway (166). The former pathway plays a predominant role in the enterocytes (167) and adipose tissue (168), while the latter is a *de novo* pathway in most tissues. These two pathways share the final step in converting diacylglycerol (DG) to TG, a reaction catalyzed by diacylglycerol acyltransferase (Dgat) (158, 169). Yamazaki et al. found that Dgat1 is located in the lumen of the endoplasmic reticulum and promotes VLDL secretion, while Dgat2 has potent Dgat activity and plays a key role in cytosolic lipid accumulation; Dgat2 overexpressing mice had a 3.1-fold increase in hepatic fat content (170). In line with this observation, in high-fat fed obese mice liver-specific Dgat2 inhibition caused a marked reduction in hepatic TG content and plasma lipid levels (171).

In the present study, Dgat1 mRNA abundance accounted for 0.15%, whereas Dgat2 mRNA abundance accounted for 4.93% of total genes measured. We observed numerical increases in the hepatic expression of Dgat1 (CLA = 0.30, control = -0.02; $p = 0.18$) and Dgat2 (0.25, control = 0.03; $p = 0.30$) in the mice fed *trans*-10, *cis*-12-CLA. Although there was substantial variation in the expression of Dgat2 among animals, it could be speculated that this increase in Dgat2 mRNA expression corresponded to an actual metabolic requirement for stimulating TG synthesis. Such response has been clearly

observed in numerous studies with *trans*-10, *cis*-12-CLA that resulted in liver enlargement (25, 28-32, 113) (See Table 4.2).

***Trans*-10, *cis*-12-CLA stimulated glyceroneogenesis in liver**

Pck1 and gluconeogenesis and glyceroneogenesis

Phosphoenolpyruvate carboxykinase 1 (Pck1) catalyzes the rate-limiting step in the gluconeogenesis pathway, converting oxaloacetate to phosphoenolpyruvate (172). In addition, Pck1 also is important for generation of G3P during the process of glyceroneogenesis which under most physiological circumstances is the predominant pathway to generate the glycerol backbone for TG synthesis (173). Pck1 mRNA abundance accounted for 14.79% of total genes measured. We observed a significant increase in hepatic Pck1 expression (CLA = -0.29, control = -1.74; $p = 0.05$) in mice fed *trans*-10, *cis*-12-CLA, which was indicative of increased glyceroneogenesis and/or gluconeogenesis in liver. Although we did not measure the plasma insulin level in mice, the *trans*-10, *cis*-12-CLA-induced hyperinsulinemia with a normal range of blood glucose level demonstrated by Clément et al. (33) would inhibit gluconeogenesis via Pck1. Furthermore, a greater degree of glyceroneogenesis would agree with the greater expression of Gpm. Therefore, this could contribute to enhanced TG synthesis and accumulation in liver due to *trans*-10, *cis*-12-CLA.

Pdk4 and glucose metabolism

The pyruvate dehydrogenase complex (PDC) catalyzes the irreversible oxidative decarboxylation of pyruvate into acetyl-CoA (174). Regulating PDC is an important step in fuel selection of energy utilization in animals during different nutritional and hormonal states as the modulation of PDC activity impacts fatty acid as well as pyruvate and glucose metabolism (175). Phosphorylation of PDC via pyruvate dehydrogenase kinase (Pdk) inhibits its activity (175, 176), thereby reducing the conversion of pyruvate to acetyl-CoA (177). Four isoenzymes of Pdk have been identified in mammalian tissues, including Pdk1, Pdk2, Pdk3 and Pdk4 (176). The abundance of the Pdk4 isoform, which is highly expressed in liver, heart and skeletal muscle, is transcriptionally controlled (178).

Pdk4 mRNA abundance accounted for < 1% of total genes measured. In our study, the group fed with *trans*-10, *cis*-12-CLA had significantly higher hepatic Pdk4 expression (CLA = 0.47, control = -1.85; $p = 0.001$) compared to the control mice, indicative of reduced glucose oxidation. Since expression of the Pdk4 gene is induced by high fat diets and long chain fatty acids (179), the results may suggest that increased Pdk4 expression could be the consequence of *trans*-10, *cis*-12-CLA intake.

***Trans*-10, *cis*-12-CLA enhanced lipid droplet formation in the liver**

Plin and lipid droplet formation

Perilipin (Plin) proteins are vital for cytoplasmic lipid droplet (LD) formation and also lipolysis (180). In isolated hepatocytes, as well as livers from mice and humans, Plin2 levels are proportional to hepatic lipid content (181); however, it is unclear if increased Plin2 is a cause or consequence of fatty liver (182). Importantly, the Plin2 knockout mice display a 60% reduction in hepatic TG and are resistant to diet-induced fatty liver (183). To determine the mechanism for the reduced hepatic TG content, that study measured hepatic lipogenesis, VLDL secretion, and lipid uptake and utilization, all of which were shown to be similar between mutant and wild-type mice (183). The finding of similar VLDL output in the presence of a reduction in total TG in the Plin2 knockout liver was explained by retention of TG in the microsomes where VLDL is assembled. Given that LDs are thought to form from the outer leaflet of the microsomal membrane, the reduction of TG in the cytosol with concomitant accumulation of TG in the microsome of Plin2 knockout cells suggested that Plin2 facilitates the formation of new LDs (183).

In our study, expression of Plin2 mRNA accounted for 1.61%. We found that the hepatic expression of Plin2 in the mice fed *trans*-10, *cis*-12-CLA was significantly up-regulated (CLA = 0.40, control = -0.43; $p = 0.0017$). Since Plin2 may play a role in facilitating the formation of new LDs (183), the up-regulation of Plin2 was suggestive of

enhanced formation of hepatic LDs in mice fed *trans*-10, *cis*-12-CLA, thereby, at least in part, contributing to TG accumulation and hepatic steatosis. This may be explained by its specific role in the early stages of lipid droplet formation (182). Therefore, we suggest that enhanced LD formation via up-regulated expression of Plin2 could be a novel mechanism of *trans*-10, *cis*-12-CLA's hepatic steatotic effect on liver.

***Trans*-10, *cis*-12-CLA increased VLDL assembly and secretion**

ApoB, Mttp and VLDL assembly/secretion

In the liver, synthesized TG is either stored in cytoplasmic droplets or secreted as VLDL particles (170). Most de novo synthesized TG is stored in cytosolic TG pools and a smaller portion is secreted in the form of VLDL. Cytosolic TG pool size has been shown to correlate with VLDL secretion. Apo-protein B 100 (ApoB) is the key component whose rate of synthesis in the rough endoplasmic reticulum controls the overall rate of VLDL production (184). Lipid components are added to apoprotein B (185) by microsomal TG transfer protein (Mttp) (186). Then the apoproteins are glycosylated in the Golgi apparatus (184) where secretory vesicles bud off, migrate to, and fuse with the membrane of the hepatocytes and release the VLDL into blood (184). Since Mttp catalyzes the transfer of lipids to the apoB molecule, Mttp may play a crucial role in the assembly and secretion of VLDL in the liver (187). Patients with abetalipoproteinemia have a defect in the Mttp gene

resulting in severely deficient lipoprotein secretion (188). Furthermore, Mttp inhibitors can reduce the production of apoB-lipoproteins in both hepatoma cell cultures (189-191) and *in vivo* in a rabbit model of human homozygous familial hypercholesterolemia (192). Minehira et al. showed that in mouse liver, mutant Mttp gene (homozygous knockout) increases hepatic steatosis in mouse (193).

Both Lin et al. and Yotsumoto et al. indicated that *trans*-10, *cis*-12-CLA reduced both apoB synthesis and TG secretion in HepG2 cells (144, 146). Several *in vivo* studies (31, 48, 113) observed reduced plasma TG levels in mice fed *trans*-10, *cis*-12-CLA, supporting the findings from *in vitro* studies. In contrast, when it comes to clinical studies, previous short-term studies in humans have revealed varying effects of CLA on blood lipids.

Whigham et al. (120) found increased plasma TG levels in subjects receiving 6 g/d of CLA mixture (*trans*-10, *cis*-12-CLA: *cis*-9, *trans*-11-CLA = 1:1) after 28 and 52 weeks of feeding compared with controls. In contrast, Iwata et al. observed no significant changes in blood TG from Japanese male volunteers receiving low or high CLA mixtures or control (119).

Another study found a reduction of VLDL-TG and TG in the blood in human subjects receiving 3 g/d of CLA mixture (*trans*-10, *cis*-12-CLA: *cis*-9, *trans*-11-CLA = 1:1) for 8 weeks (194).

In our study, Apob mRNA abundance accounted for 9.14% and Mttp mRNA abundance accounted for 1.24% of total genes measured; however, we observed a numerical

increase in Apob expression (CLA = -0.05, control = -0.33; $p = 0.29$) and a significant up-regulation of expression of Mttp (CLA = 0.14, control = -0.37; $p = 0.04$) in the mice fed *trans*-10, *cis*-12-CLA. Despite the apparent accumulation of TG in the liver (24) leading to greater weight (Table 4.1), our results suggest that the VLDL assembly/secretion mechanism in mice fed *trans*-10, *cis*-12-CLA was not impaired and may have actually been enhanced via the up-regulation of hepatic Mttp. It was reported previously that in *trans*-10, *cis*-12-CLA-fed mice the VLDL secretion rate was increased; whereas, the plasma TG concentration was decreased (30). Together with our results, studies suggest that TG-rich lipoproteins and NEFAs are taken up in the *trans*-10, *cis*-12-CLA-fed animals to such an extent that the VLDL secretion rate, even when increased, is insufficient to eliminate excess fatty acid (Table 4.1) entering the whole esterification and VLDL assembly/secretion pathways, which would account for part of the fat deposit within liver cells.

Furthermore, our findings were in agreement with recent kinetic studies. They have pointed out that the actual amount of hepatic TG-rich lipoprotein secretion rate in non-alcoholic fatty liver disease is actually increased, but is inadequate to match the increased TG synthesis in the liver (195). One of those studies found that subjects with NAFLD failed to further increase VLDL-TG secretion rate when hepatic TG infiltration exceeded 10% suggesting the liver of these subjects had a limited capacity to secrete VLDL particles (195).

Cideb and VLDL secretion

The cell death-inducing DFF45-like effector (Cide) family of proteins consists of three molecules, including Cidea, Cideb and Cidec/fat specific protein 27 (Fsp27) (196). Mouse Cideb is mainly expressed in liver and kidney (197). Recently, Li et al. observed that liver of Cideb null mice had higher levels of TG accompanied by lower levels of VLDL secretion (198). They also found that Cideb is localized to smooth ER and LDs and identified apoB as a Cideb-interacting protein (198). By infecting adenoviruses expressing various Cideb truncations into hepatocytes of Cideb-null mice, they found that Cideb requires both its apoB-binding and LD association domains to restore the secretion of TG-enriched VLDL particles (198). Their data suggest that Cideb promotes the formation of TG-enriched VLDL particles (198).

Cideb mRNA abundance accounted for 3.46% of total genes measured in the present study and its expression in mice fed *trans*-10, *cis*-12-CLA was significantly up-regulated (CLA = -0.04, control = -1.13; $p = 0.05$). The greater expression of Cideb in the liver suggested more effective TG secretion via VLDL and underscored that hepatic steatosis induced by *trans*-10, *cis*-12-CLA was not due to impaired VLDL secretion. However, given the increased liver weight, the increase in VLDL secretion might be insufficient to eliminate the fatty acid (Table 4.1) entering the whole esterification and VLDL assembly/secretion pathways.

Transcription regulation and lipogenesis and TG synthesis

Srebf1-related networks and TG synthesis

Sterol regulatory element binding transcription factor 1 (Srebf1), also known as sterol regulatory element-binding protein 1 (Srebp1), is a transcription factor involved in hepatic lipogenesis and also adipocyte differentiation (199, 200). It is predominantly expressed in liver and adipose tissue (201-203). Srebf1 is synthesized as a precursor protein and is released from the ER membrane into the nucleus as a mature protein by a sequential cleavage process to bind sterol regulatory elements and then activate expression of the target genes (204, 205), such as *Acaca* (206) and *Scd1* (207). Overall, Srebf1 plays a central role in energy homeostasis by promoting glycolysis, lipogenesis, and adipogenesis (208).

Srebf1 mRNA abundance accounted for 0.67% of total genes measured in our study. The hepatic Srebf1 expression in mice fed *trans*-10, *cis*-12-CLA was significantly up-regulated (CLA = 0.14, control = -0.22; $p=0.02$), which was in agreement with previous published reports indicating that a CLA-mix diet significantly induced the Srebf1 expression in the murine liver (33, 209). In freshly-isolated hepatocytes, Srebf1c mRNA was activated by insulin (210). Thus, the up-regulated Srebf1 gene in our study may have been a response to higher levels of insulin in mice fed *trans*-10, *cis*-12-CLA which has been previously reported by Clément et al, Halade et al. and Tsuboyama-Kasaoka et al (22, 33, 48). In our study, the expression of Srebf1's target genes, including *Acaca*, *Scd*, and *Gpam*

(206), were all significantly up-regulated in mice fed *trans*-10, *cis*-12-CLA, which suggests that the liver was generating more palmitic acid, oleic acid and TG (211). Collectively, these results indicate that *trans*-10, *cis*-12-CLA stimulates the lipogenic pathway by indirectly up-regulating *Srebf1*, leading to an overall increase in fat accumulation in liver.

Chrebp and lipid metabolism

Carbohydrate response element binding protein (Chrebp) is a transcription factor responsible for glucose-induced transcription of liver pyruvate kinase (LPK) gene (212). Some studies provided evidence for a direct and dominant role of Chrebp in the glucose regulation of two key liver lipogenic enzymes, *Acaca* and *Fasn*, therefore Chrebp is also known to be major regulator of lipogenic enzymes (157, 213). Chrebp is expressed constitutively in hepatocytes (214) and is known to recognize E box sequences in the promoters of target genes (215). The regulation of Chrebp involves phosphorylation-dependent mechanisms responsive to feeding (glucose and fatty acids) and fasting (glucagon) (216). Under low glucose conditions, Chrebp remains in the cytosol and unable to bind DNA as the result of phosphorylation at multiple sites (214). The dephosphorylation of Chrebp in response to high glucose does not require insulin (213).

In our study, Chrebp mRNA was numerically up-regulated (CLA = -0.17, control = -0.22; $p = 0.62$). Chrebp mRNA abundance accounted for 2.83% of total genes measured.

However, some of its target genes involved in lipid metabolism in the liver were significantly up-regulated, including *Acaca* and *Gpam*, suggesting that *trans*-10, *cis*-12-CLA indirectly stimulated lipogenic genes through *Chrebp* as well as *Srebf1*.

Ppara and lipid catabolism

The ligand-activated transcription factor, peroxisome proliferator-activated receptor α (*Ppara*), modulates lipid catabolism via specific *Ppar* responsive elements (PPREs) in the regulatory regions of genes such as lipoprotein lipase (134), *Acox1* (217), *Cpt1*, thioesterases, fatty acid binding protein, peroxisomal and mitochondrial β oxidation enzymes, microsomal ω -oxidizing enzymes and apolipoproteins (131). Peroxisome proliferators (PPs) activate *Ppara* which then heterodimerises with RXR and binds to DNA at PPREs upstream of PP responsive genes (218). Activation of *Ppara* by ligands results in altered transcription of many of these genes which may stimulate fatty acid uptake and activation, mitochondrial oxidation, peroxisomal fatty acid oxidation, ketogenesis, and fatty acid elongation and desaturation (219).

Isomers of CLA have some structural features similar to PPs and importantly the physiological responses observed in mice (reduced body weights, hepatic lipid accumulation, hypolipidemia) are characteristic of this group of chemicals (134). As *trans*-10, *cis*-12-CLA has been reported to be a potent ligand of *Ppara* (134), the underlying

regulations might imply the activation of Ppara. In our study, the hepatic Ppara expression (CLA = -0.19, control = -0.33; p = 0.42) in mice fed *trans*-10, *cis*-12-CLA was numerically higher than in the control group. Ppara mRNA abundance accounted for 1.28% of total genes measured. Although no significantly up-regulation of Ppara was observed in our study, many of its target genes playing important roles in lipid metabolism (Cd36, Plin2, Scd, Pck1, Pdk4, Acox1, Ucp2 and Srebf1) were significantly up-regulated, providing indirect evidence that *trans*-10, *cis*-12-CLA might act through Ppara.

Despite dietary *trans*-10, *cis*-12-CLA induced expression of Ppara target genes, including fatty acid transporter, Srebp1 and Fasn genes, *in vitro* transactivation assays demonstrated that *trans*-10, *cis*-12-CLA and *cis*-9, *trans*-11-CLA were equally efficient at activating Ppara, thus the specific effect of *trans*-10, *cis*-12-CLA is unlikely to result from direct interaction with this nuclear receptor (33). In another study, wild-type or Ppara-null mice were fed a diet containing 0.5% CLA mixture (*trans*-10, *cis*-12-CLA represented 43.5% of CLA). After 4-week feeding period, while CLA feeding resulted in specific activation of Ppara target genes in liver that contribute to increased use of fatty acids as substrates and subsequent reductions in body weight/fat content, changes in those Ppara target gene expression were found in both wild-type and Ppara-null mice fed CLA (125) (125).

Apart from those, numerous lines of evidence indicate that it has a major role in

glucose metabolism (220). First, several studies (221-223) showed that fasting Ppar α -null mice display marked hypoglycemia. Second, both Guerre-Millo et al. and Tordjman et al. found that high-fat feeding-induced insulin resistance in mice was mitigated in the absence of Ppar α (224, 225). Third, a study found that induction of the gluconeogenic genes Pck1 and glucose 6-phosphatase by dexamethasone is Ppar α dependent (226), although they are not direct target genes of Ppar α (220). Fourth, Patsouris et al. showed that Ppar α decreases plasma glycerol levels in mice and humans by directly upregulating the expression of genes involved in hepatic gluconeogenesis from glycerol, including cytosolic glycerol 3-phosphate dehydrogenase (cGpdh), mitochondrial glycerol 3-phosphate dehydrogenase (mGpdh), glycerol kinase, aquaporin 3 (AQP3), and aquaporin 9 (AQP9) (220). They also pointed out that the stimulatory effect of Ppar α on gluconeogenic gene expression is associated with elevated hepatic glucose production during fasting (220). Above all, it can be concluded that Ppar α has an important influence on glucose metabolism.

Trans-10, cis-12-CLA increased fatty acid oxidation in the liver

Since the decrease in fatty acid oxidation may cause fat accumulation in the liver (31), it is intuitive that the mRNA expression of the enzymes related to fatty acid oxidation may be down-regulated during liver lipodosis. However, recent investigations of the *trans-10, cis-12-CLA*'s effects on fatty acid oxidation are controversial. Rasooly et al.

indicated that *trans*-10, *cis*-12-CLA supplementation decreased the expression of genes involved in fatty acid oxidation, including flavin monooxygenase-3 (FMO3) (95%, $p < 0.0001$), cytochrome P450 (cyt P450) (61%, $p = 0.002$), Cpt1a (77%, $p = 0.025$), acetyl CoA oxidase (50%, $p = 0.08$) and Ppara (65%, $p = 0.05$) (227). However, in the study of Gruffat et al., liver tissue samples of rats incubated with a fatty acid mixture (representative of circulating NEFA) and *trans*-10, *cis*-12-CLA or oleate they found that the rate of CLA isomer oxidation was two times higher than that of oleate ($p < 0.01$) (150). Furthermore, expression of mice hepatic fatty acid oxidation genes also increased in three studies with a mixture of CLA isomers (124, 129, 228) and in one study with *trans*-10, *cis*-12-CLA (31).

Cpt1 and fatty acid oxidation

Cpt1 is considered the rate-limiting enzyme for mitochondrial β oxidation (227). Degrace et al. found an increase in the activity of hepatic Cpt1 in mice supplemented with *trans*-10, *cis*-12-CLA accompanied by an increase in Acox1 activity and gene expression (31). Another study conducted by Viswanadha et al. noted that *trans*-10, *cis*-12-CLA increased the oxidation of fatty acids in murine hepatic cell lines, as indicated by increased expression of mRNA for Cpt1 (229). In our study, *trans*-10, *cis*-12-CLA supplementation the hepatic expression of Cpt1a (CLA = -0.32, control = -0.43; $p = 0.57$) was numerically greater with CLA. Cpt1a mRNA abundance accounted for 1.91% of total genes measured.

Moreover, malonyl-CoA plays a crucial role in the control of mitochondrial fatty acid oxidation through its inhibition of Cpt1 (230). As mentioned in the section discussing Acaca and de novo fatty acid synthesis, more malonyl-CoA was generated by up-regulated Acaca expression in liver in response to *trans*-10, *cis*-12-CLA. As a result, Cpt1 expression was supposed to down-regulated or inhibited. However, unexpectedly we observed a numerically upregulation of Cpt1 expression in liver.

Acox1 and fatty acid oxidation

Peroxisomes play an important role in lipid catabolism, since very long chain fatty acids (VLCFAs) are almost exclusively processed by the peroxisomal β oxidation system, whereas fatty acids up to 18 carbons in length are oxidized by the mitochondria (231). Acyl-CoA oxidase 1 (Acox1) catalyzes the first and rate-limiting enzyme of the peroxisomal fatty acid β oxidation pathway of VLCFAs (232). The reduction of the activity and/or mRNA of fatty acid oxidation enzymes in peroxisomes could contribute to the lipid accumulation in hepatocytes and to the severity of steatohepatitis. Fan et al. found that mice lacking the peroxisomal Acox1 gene developed severe steatohepatitis, lipogranulomas and hepatocellular carcinomas (233). Acox1 mRNA abundance accounted for 6.83% of total genes measured in our study and we found that mice fed *trans*-10, *cis*-12-CLA had greater Acox1 expression (CLA = 0.13, control = -0.22; $p = 0.05$), suggesting increased fatty acid β

oxidation in peroxisomes. The up-regulation of Acox1 might be due to the relatively lower amount of dietary *trans*-10, *cis*-12-CLA supplementation compared with previous studies (22, 23, 28, 32, 34-36).

A study conducted by Clément et al. showed that mice fed a diet enriched in 0.4% (w/w) *trans*-10, *cis*-12-CLA for 4 weeks developed fatty liver and observed a dramatic increase in non fasting plasma insulin levels which were 10 times higher in these mice than those fed control (33). Despite this marked hyperinsulinemia, they also pointed out that plasma glucose concentration remained within the normal range in these mice fed *trans*-10, *cis*-12-CLA (33). Thus, they suggest that the train of events leading to *trans*-10, *cis*-12-CLA-induced alterations in liver is probably indirect and that the liver steatosis occurring in *trans*-10, *cis*-12-CLA-fed mice is secondary to hyperinsulinemia which causes high levels of fatty acid uptake and synthesis (33).

In our study, the up-regulation of Acox1 coupled with the lack of change in Cpt1 mRNA expression indicated that decreased fatty acid oxidation could not be the factor triggering hepatic steatosis (Table 4.1), but might be a consequence of it. Under hyperinsulinemia condition, the effect of elevated β oxidation due to *trans*-10, *cis*-12-CLA was overwhelmed by an increase in uptake of fatty acids as well as greater lipogenesis in liver, thereby resulting in hepatic steatosis as observed previously (24).

Trans-10, cis-12-CLA stimulated ketogenesis in the liver

Hmgcs2 and ketogenesis

Under conditions of increased fatty acid uptake and low blood insulin to glucagon ratio, the liver often produces large amounts of the ketone bodies, acetoacetate and β -hydroxybutyrate, in a process known as ketogenesis (184). Moreover, ketogenesis is controlled indirectly by Cpt1 (234) and directly by the activity of the mitochondrial key regulatory enzyme 3-hydroxy-3-methylglutaryl-CoA synthase 2 (Hmgcs2) (235).

Conversion of acetyl-CoA to ketone bodies, rather than complete oxidation in the TCA cycle, results in the formation of less ATP/mole of fatty acid oxidized, e.g. five time less in the case of palmitate (129 vs. 27 ATP/mole, TCA and oxidative phosphorylation in the electron transport chain vs. conversion of acetyl-CoA to ketone bodies) (184). Then why liver produces ketone bodies? Ketogenesis allows the liver to metabolize about five times more fatty acids (for the same ATP yield) and synthesize water-soluble fuels that other tissues can use when there is little glucose available (184).

In the present study, Hmgcs2 mRNA abundance accounted for 13.88% of total genes measured, which was the second highest gene expression among all the genes determined. Our results support previous findings of greater hepatic Hmgcs2 expression (CLA = 0.13, control = -0.16; $p = 0.05$) in mice fed *trans*-10, *cis*-12-CLA, suggesting that this CLA isomer induces more ketone body generation in liver, which was in agreement

with a previous study showing that the liver of rats fed CLA produced significantly more ketone bodies than those of rats fed a linoleic acid-rich diet (236).

Bdh1 and ketone body utilization

3-hydroxybutyrate dehydrogenase 1 (Bdh1) plays a key role in redox balance and energy metabolism since in the presence of NADH, the hepatic Bdh transforms acetoacetate into D-3-hydroxybutyrate, which is then transported through the blood stream to peripheral tissues, e.g., brain, heart, and kidney (237). Bdh1 mRNA abundance accounted for 3.35% of total genes measured. We observed that Bdh1 expression in mice fed *trans*-10, *cis*-1-CLA (CLA = 0.13, control = -0.20; $p = 0.05$) was significantly higher compared with the control. The increase in Bdh1 expression suggested that more 3-hydroxybutyrate was produced from acetoacetate, which was supported by previous findings showing that liver from CLA-fed rats produced significantly more ketone bodies and the ratio of 3-hydroxybutyrate to acetoacetate was consistently higher in the liver perfusate (236).

Together with data on Hmgcs2 this was indicative of stimulated ketone body production in liver. Indirectly, expression of Hmgcs2 and Bdh1 would support the notion that there likely was greater adipose tissue lipolysis in these mice, thus, providing ample NEFA for oxidation and ketogenesis. The results suggest that probably due to high rate of fatty acid oxidation in the liver, increased generation of acetyl CoA exceeded the capacity of

the TCA cycle; as a result, acetyl CoAs were used for ketogenesis and thereby leading to increased expression of Hmgcs2 and Bdh1 expression in liver.

Trans-10, cis-12-CLA changes energy metabolism in liver

Ucp2 and energy metabolism

Uncoupling proteins (Ucps) belong to a family of mitochondrial anion carriers and are present in the mitochondrial inner membrane (238). Ucps dissipate the proton gradient by allowing the re-entry of protons into the mitochondrial matrix during oxidative ATP generation, resulting in the uncoupling of the respiratory chain and heat production (239). Ucp2 isoform shows the widest tissue expression of all Ucps including liver, skeletal muscle, heart and kidney (240). *In vivo* studies indicate that physiological and pathological elevation of blood LCFAs resulting from fasting (241-243) or high fat diet (244) induce upregulation of Ucp2. Peters et al. demonstrated that dietary CLA increases gene expression of Ucp2 in the liver of C57BL/6N mice (125). Ucp2 mRNA abundance accounted for 0.19% of total genes measured. Our study observed an upregulation of hepatic Ucp2 expression (CLA = 0.15, control = -0.31; $p = 0.002$) in mice fed *trans-10, cis-12-CLA* suggesting that the increased Ucp2 mRNA expression may serve as a counterregulatory mechanisms to lower cellular ATP levels and decrease metabolic efficiency, thereby reducing fat accumulation in the long-term (240).

***Trans*-10, *cis*-12-CLA and stress response**

According to the complex mechanisms proposed by Jaudszus et al. (5), the anti-adipogenic effect of *trans*-10, *cis*-12-CLA may be primarily the consequence of pro-apoptotic and pro-inflammatory responses in adipose tissue, including the nuclear factor κ B (NF κ B)-dependent production of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and interleukin-8 (IL-8) (21, 22, 245). These cytokines produced by adipose tissue induce the expression of the proteins suppressor of cytokine signaling-3 (SOCS-3) and JNK, which can inhibit insulin signal transduction leading to hyperinsulinemia and insulin resistance (21), thereby suppressing lipid synthesis and increasing lipolysis in adipocytes. The *trans*-10, *cis*-12-CLA-mediated suppression of the transcription factor PPAR γ in the adipose tissue was suggested to be indirectly responsible for the pro-inflammatory conditions (21, 246).

In our study, the genes associated with inflammation or stress response as listed in Table 7 were all numerically up-regulated, a response likely related to the relatively low dosage of *trans*-10, *cis*-12-CLA that was fed.

Summary

In our study the excess fatty acid accumulation and greater liver mass in growing male mice consuming *trans*-10, *cis*-12-CLA seems to be attributed to increased fatty acid

uptake, stimulated TG synthesis, and enhanced LD formation in the liver. Guillén et al. observed a significant increase in hepatic Cd36 expression in mice fed *trans*-10, *cis*-12-CLA and demonstrated a significant association between Cd36 expression and the degree of hepatic steatosis. Our finding of up-regulation of Cd36 expression in the liver supported those earlier results. Hence, the increased hepatic fatty acid uptake could be one potential mechanism by which *trans*-10, *cis*-12-CLA causes hepatic steatosis, i.e. the peripheral action of *trans*-10, *cis*-12-CLA leading to adipose tissue catabolism provides NEFA which must be metabolized by tissues and primarily liver.

The greater up-regulation of *Acaca*, *Gpam* and *Scd* mRNAs indicates that increased TG synthesis in the liver contributes to hepatic steatosis in mice fed *trans*-10, *cis*-12-CLA. Furthermore, the up-regulation of *Acaca* indicated that the increased hepatic lipogenesis would be used for biosynthesis of TG via *Gpam*. Hepatic lipogenesis encompasses a complex series of reactions occurring in part in the cytosol and the mitochondrial matrix (247). Ferramosca et al. found that the activity of mitochondrial citrate carrier (*Cic*), considered as the bridge connecting these two different cellular compartments, was stimulated by a CLA mixture. The consequent increase in citrate efflux from mitochondria would supply the cytosol with the carbon source, in the form of acetyl-CoA for hepatic lipogenesis (247).

We also observed a potential for enhanced lipid droplet formation in the liver of

the mice fed *trans*-10, *cis*-12-CLA. The up-regulation of Plin2 which is vital for cytoplasmic lipid droplet formation, suggests that the steatotic effect of *trans*-10, *cis*-12-CLA may, in part, be achieved by increasing the lipid storage capacity of hepatocytes. Therefore, it is suggested that increased fatty acid uptake, stimulated TG synthesis, and enhanced lipid droplet formation might together contribute to the hepatic steatotic effect of *trans*-10, *cis*-12-CLA.

The mechanisms by which the liver becomes steatotic in response to *trans*-10, *cis*-12-CLA appear to be complex, since our study found that *trans*-10, *cis*-12-CLA induced β -oxidation in the peroxisome without changing it in the mitochondria as evidenced by the up-regulation of Acox1 and lack of effect on Cpt1. Induced β -oxidation would not be associated with lipid accumulation. There are two possibilities to explain these scenarios. First, induced β -oxidation in peroxisomes might be a consequence of excess fat accumulation in the liver and a favorable mechanism to help consume excess fatty acid influx into liver because expression of Pnpla2 (a TG lipase) was numerically greater with *trans*-10, *cis*-12-CLA. Second, the relatively low level of supplementation of *trans*-10, *cis*-12-CLA was not enough to impair hepatic β -oxidation which has been observed in the study (127) conducted by Rasooly et al.

Apart from β -oxidation, reduced VLDL assembling/secretion would not be a factor triggering hepatic steatosis in the mice fed *trans*-10, *cis*-12-CLA. In our study, the

expression of Mttp, which plays a crucial role in the assembly and secretion of VLDL in the liver, was up-regulated in the mice fed with *trans*-10, *cis*-12-CLA; and, hepatic CIDEA expression was significantly higher in the mice fed with *trans*-10, *cis*-12-CLA than that in control mice, indicating more effective TG secretion via VLDL. Thus, hepatic steatosis in the mice fed with *trans*-10, *cis*-12-CLA would not be due to impaired VLDL assembling/secretion.

The increased VLDL assembling/secretion could be a result of excess TG accumulation in liver and the subsequent lipolysis via Pnpla2. However, there is only one possibility that the increased VLDL assembling/secretion could be a triggering factor of fatty liver, when the VLDL overproduction from the enlarged liver was insufficient to eliminate the flux of fatty acids entering the whole esterification and lipoprotein assembling/secretion pathway. After being absorbed in hepatocytes, fatty acids must be therefore directed towards the esterification pathway. As a result, the increase in Dgat mRNA expression in the liver of *trans*-10, *cis*-12-CLA-fed mice corresponded to an actual metabolic requirement for meeting the excessive flux of fatty acids, which was in agreement with the findings from Degraze et al. They also indicated that the flux of lipids permanently cleared from blood and taken up more abundantly by a heavier liver was possibly the starting point of the observed inductions (30).

Table 4.1 Liver weight and fatty acid content for male mice fed trans-10, cis-12-CLA (24)

	Dietary <i>trans</i> -10, <i>cis</i> -12-CLA			
	0%	0.30%	SEM	P < *
Liver weight (g)	1.99	2.36	0.12	0.04
Adipose weight (g)	0.61	0.18	0.05	0.0001
Total fatty acids (μ g/mg)	38.8	59.0	3.0	0.01

* Probability of a linear effect due to dietary trans-10, cis-12-CLA content.

Table 4.2 Summary of mouse studies including liver weight, liver TG, adipose tissue measurements in response to *trans*-10, *cis*-12-CLA

Reference	Dose	Duration	<i>trans</i> -10, <i>cis</i> -12-CLA*		
			Liver weight	Liver TG	Adipose tissue
Clément et al. (2002) (33)	0.4% (w/w)	4 wks	3.1-fold ↑		85% ↓
Degrace et al. (2003) (30)	10 g	4 wks	60% ↑	655% ↑	83% ↓
Warren et al. (2003) (113)	0.5%	8 wks	98% ↑	472% ↑	59% ↓
Degrace et al. (2004) (31)	1%	4 wks	80% ↑	646% ↑	85% ↓
Kelley et al. (2004) (25)	0.5%	8 wks		30% ↑	
House et al. (2005) (248)	1%	2 wks	33% ↑	61% ↑ (fat)	58% ↓
Viswanadha et al. (2006) (24)	0.30%	6 wks	17% ↑	52% ↑ (total fatty acids)	81% ↓
Halade et al. (2009) (48)	0.5%	0.5 y	50% ↑		73% ↓
Ashwell et al. (2010) (32)	1%	2 wks	33% ↑	62.5% ↑ (fat)	

* The increase or decrease in percentage was calculated by (CLA – Control) / Control.

Table 4.3 Hepatic expression of genes related to lipogenesis and carbohydrate metabolism following *trans* -10, *cis* -12-CLA consumption by growing male mice

Gene	Treatments ¹		Time		SEM ²
	Control	CLA	Week 2	Week 6	
<i>Carbohydrate Metabolism</i>					
<i>Gck</i>	-0.45	-0.49	-0.31	-0.63	0.28
<i>Pc</i>	-0.31	-0.08	-0.08	-0.32	0.28
<i>Pck1</i>	-1.74 ^a	-0.29 ^b	-0.80	-1.24	0.62
<i>Slc2a1</i>	-0.07	0.21	0.18	-0.04	0.14
<i>Liver TG Synthesis & Transport</i>					
<i>Apob</i>	-0.33	-0.05	-0.09	-0.29	0.19
<i>Cideb</i>	-1.13 ^a	-0.04 ^b	-0.35	-0.81	0.50
<i>Dgat1</i>	-0.02	0.30	0.23	0.049	0.17
<i>Dgat2</i>	0.03	0.25	0.26	0.02	0.15
<i>Gpam</i>	-0.31 ^a	0.44 ^b	0.13	0.004	0.17
<i>Mttp</i>	-0.37 ^a	0.14 ^b	0.002	-0.22	0.17
<i>Plin2</i>	-0.43 ^a	0.40 ^b	-0.01	-0.02	0.18
<i>Scd</i>	-0.92 ^a	0.05 ^b	-0.50	-0.37	0.19
<i>De novo Fatty Acid Synthesis</i>					
<i>Acaca</i>	0.22 ^a	0.72 ^b	0.42	0.51	0.11
<i>Lipolysis</i>					
<i>Lipc</i>	-0.95	-0.20	-0.15	-0.10	0.54
<i>Pnpla2</i>	-0.04	0.14	0.24	-0.13	0.24
<i>Transcription Regulation</i>					
<i>Chrebp</i>	-0.22	-0.17	-0.19	-0.19	0.08
<i>Srebf1</i>	-0.22 ^a	0.14 ^b	0.04	-0.11	0.11

¹Means with different superscripts differ ($P \leq 0.05$).

²SEM = the largest standard error of the mean is shown.

Table 4.4 Hepatic expression of genes related to lipid catabolism following *trans* -10, *cis* -12-CLA consumption by growing male mice

Gene	Treatments ¹		Time		SEM ²
	Control	CLA	Week 2	Week 6	
<i>Fatty Acid Oxidation & Energy Metabolism</i>					
<i>Aacs</i>	-0.21	-0.29	-0.26	-0.25	0.31
<i>Acox1</i>	-0.22 ^a	0.13 ^b	-0.08	-0.0032	0.14
<i>Bdh1</i>	-0.20 ^a	0.13 ^b	0.06	-0.12	0.12
<i>Cpt1a</i>	-0.43	-0.32	-0.36	-0.39	0.14
<i>Insig1</i>	-0.79	0.35	-0.23	-0.20	0.45
<i>Mlycd</i>	0.00054	-0.17	-0.07	-0.11	0.10
<i>Pdk4</i>	-1.85 ^a	0.47 ^b	-0.81	-0.57	0.50
<i>Ucp2</i>	-0.31 ^a	0.15 ^b	-0.04	-0.11	0.10
<i>Ketogenesis</i>					
<i>Hmgcs2</i>	-0.16 ^a	0.13 ^b	0.10 ^a	-0.13 ^b	0.10
<i>Fatty Acid Uptake</i>					
<i>Cd36</i>	-1.50 ^a	1.11 ^b	-0.01	-0.35	0.55
<i>Slc27a2</i>	-0.23	-0.04	-0.07	-0.20	0.18
<i>Transcription Regulation</i>					
<i>Ppara</i>	-0.33	-0.19	-0.23	-0.30	0.12

¹Means with different superscripts differ ($P \leq 0.05$).

²SEM = the largest standard error of the mean is shown.

Table 4.5 Hepatic expression of genes related to stress following *trans* -10, *cis* -12-CLA consumption by growing male mice

Gene	Treatments ¹		Time		SEM ²
	Control	CLA	Week 2	Week 6	
<i>Inflammation</i>					
<i>Saa1</i>	-0.50	-0.21	-0.17	-0.54	0.31
<i>Stress Response</i>					
<i>Angptl3</i>	-0.54	0.07	-0.26	-0.21	0.42
<i>Angptl4</i>	0.01	-0.29	-0.53	0.25	0.35
<i>Atf6</i>	-0.27	0.17	0.16	-0.26	0.46
<i>Ddit3</i>	-0.25	0.37	-0.11	0.24	0.30
<i>Eif2ak3</i>	-0.55	-0.10	-0.32	-0.32	0.46
<i>Fgfr2</i>	-0.09	0.19	-0.58	0.68	0.49
<i>Hspa1b</i>	-1.04	-0.54	-0.91	-0.67	0.62
<i>Xbp1</i>	-1.15	-0.55	-0.46	-1.23	0.63

¹Means with different superscripts differ ($P \leq 0.05$).

²SEM = the largest standard error of the mean is shown.

Table 4.6 Summary of various hepatic measurements in response to *trans*-10, *cis*-12-CLA

Reference	Subject	Dosage*	Liver							
			Wt	TG	Protein	FFA	VLDL secretion	Ketone body production	BHBA/AA	TG secretion
Degrace et al. (2003) (30)	Mice	10 g	60%↑	655% ↑			2-fold↑			
Kelley et al. (2004) (25)	Mice	0.5%		432%↑		↑				
Degrace et al. (2004) (31)	Mice	1%	80%↑	646%↑	19%↓	44%↓				
Sakono et al. (1999) (236)	Rat	1% CLA Mix	—	—				↑	↑	
Lin et al. (2001) (249)	HepG2 cell	1 mmol/l		4-fold↑						↓

* Without special indication (e.g. CLA Mix), dosage means the amount of *trans*-10, *cis*-12-CLA used.

Table 4.7 Summary of various serum measurements in response to *trans*-10, *cis*-12-CLA

Reference	Subject	Dosage*	Serum						
			Glucose	Insulin	Insulin resistance	CHL	TG	ApoB	NEFA
Clément et al. (2002) (33)	Mice	0.4% (w/w)	—	10-fold↑					
Halade et al. (2009) (48)	Mice	0.5%	31%↑	51%↑			20%↓		19%↑
Degrace et al. (2003) (30)	Mice	10 g					67%↓	53%↓	
Degrace et al. (2004) (31)	Mice	1%					64%↓		44%↓
Peters et al. (2001) (125)	Mice	0.5% CLA Mix	—			—	49%↓		
Tsuboyama-Kasaoka et al. (2000) (22)	Mice	1% (w/w)	—	↑	↑				

* Without special indication (e.g. CLA Mix), dosage means the amount of *trans*-10, *cis*-12-CLA used.

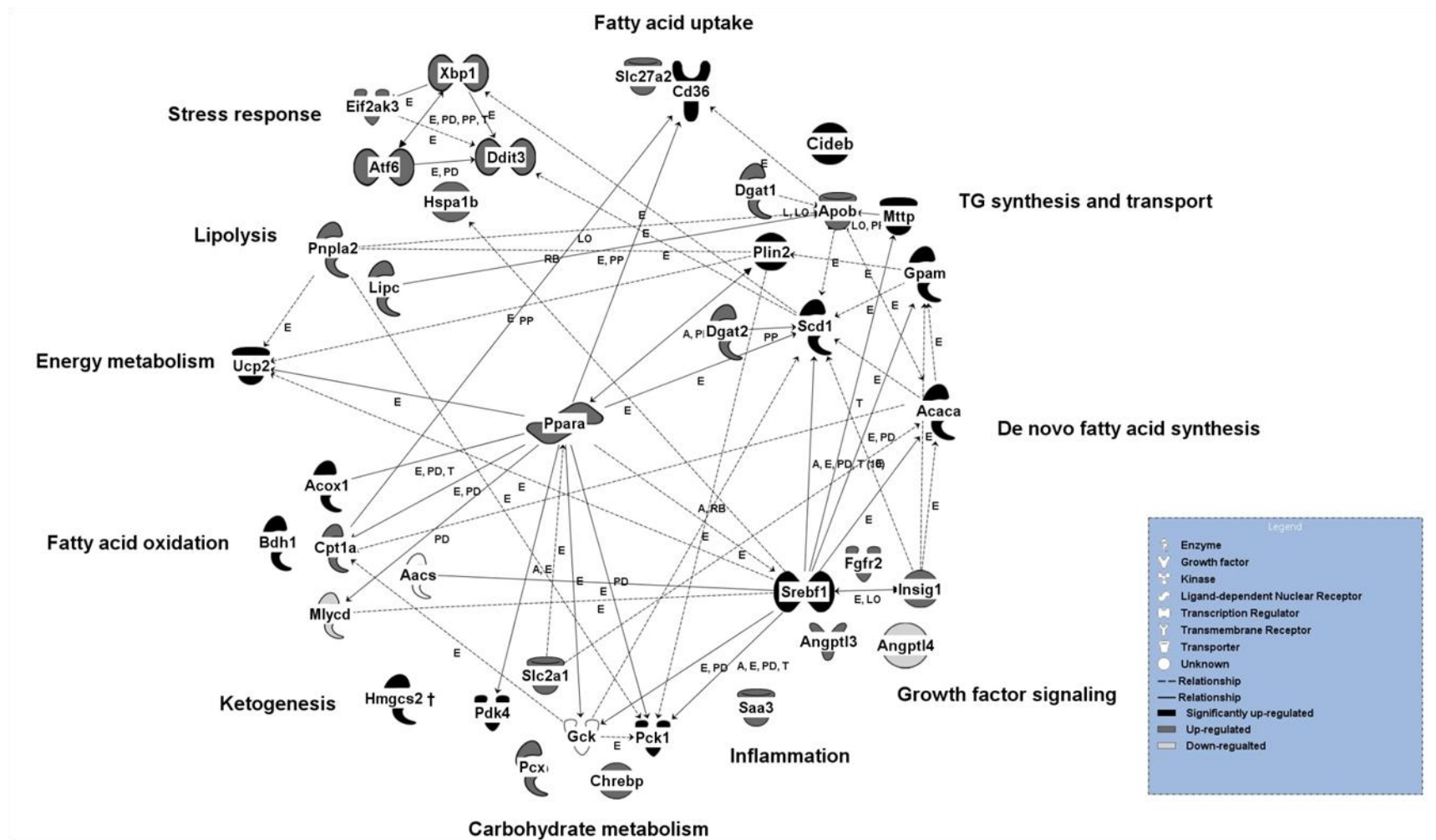


Figure 4.1 Currently known relationships among genes analyzed based on manually curated examination of the published literature within the Ingenuity Pathway Analysis (www.ingenuity.com) knowledge base. Genes are grouped by the predominant process they play in lipid metabolism. Different shapes denote the type of protein encoded by the specific genes, including enzymes, ligand-dependent nuclear receptors, transcription regulators, and transporters. Letters along the edges denote effects on activity (A), expression (E), localization (LO), proteolysis (L), RNA binding (RB), protein-DNA binding (PD), and protein-protein binding (PP). Gene names are as in Table 1.1.

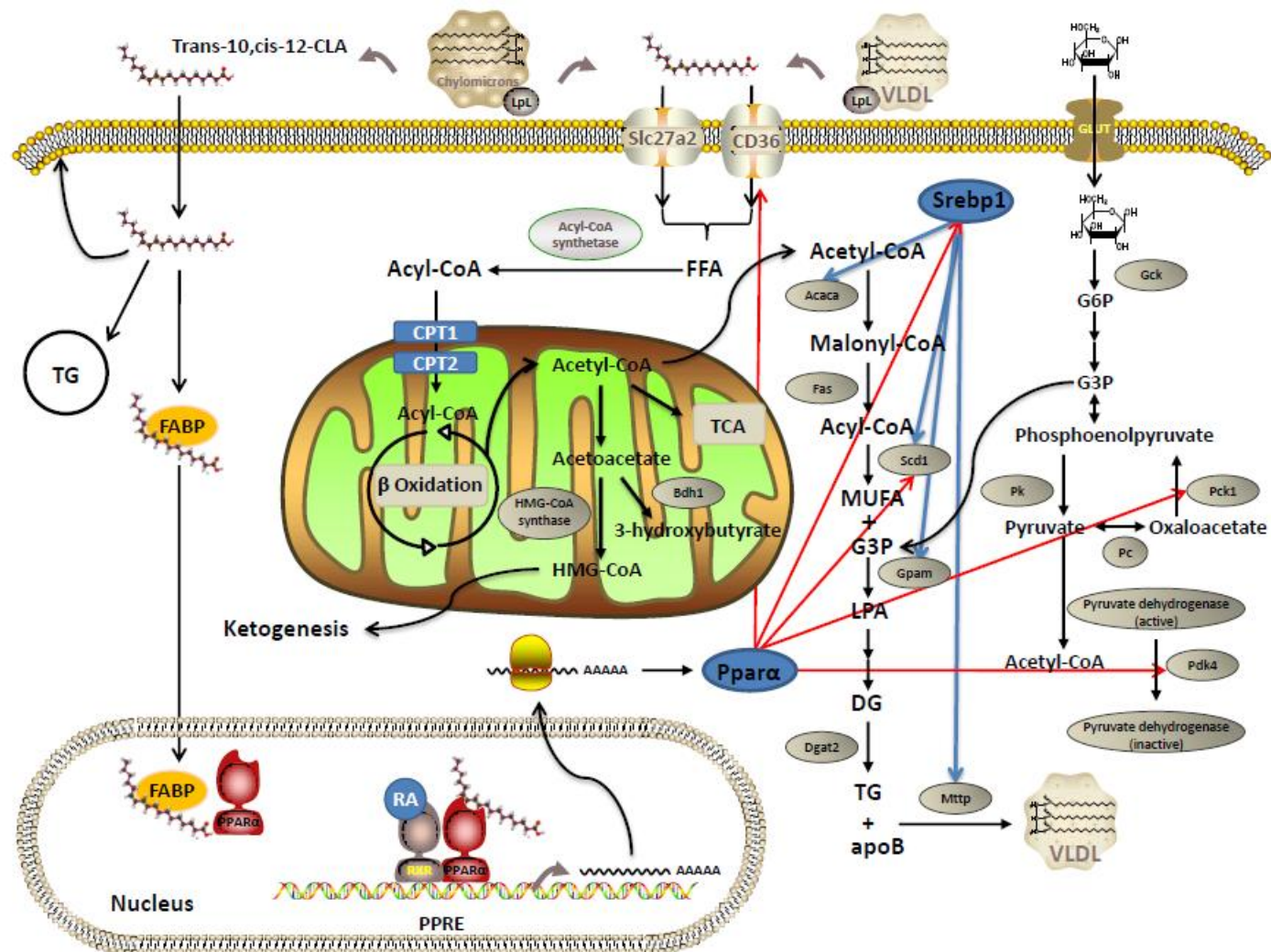


Figure 4.2 Interrelationships among cellular pathways regulating lipid and carbohydrate metabolism in the mouse liver.

CHAPTER 5

Conclusions

All in all, our study found that the hepatic steatotic effect of *trans*-10, *cis*-12-CLA might be due to increased fatty acid uptake, stimulated TG synthesis, enhanced storage capacity of lipid droplets as well as insufficiently induced VLDL assembly/secretion mechanism in the liver. Impaired β -oxidation would not be a factor contributing to hepatic steatosis induced by *trans*-10, *cis*-12-CLA. Our results provide new evidences to advance in the knowledge of the effect of this supplement on hepatic gene expression in a model of growing male mice. Furthermore, since the growing male mice was fed relatively low amount of *trans*-10, *cis*-12-CLA for 6 weeks, it seems to be a more reasonable model to speculate *trans*-10, *cis*-12-CLA's long-term effect on hepatic steatosis in humans. However, we did not capture the entire picture of the molecular mechanisms, since we did not measure gene product activity.

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APPENDIX A

Supplementary Tables

Table A.1 qPCR performance among the thirty-eight genes measured in liver.

Gene	Median Ct ¹	Median Δ Ct ²	Slope ³	(R ²) ⁴	Efficiency ⁵
<i>Aacs</i>	24.6	3.0	-3.91	0.991	1.80
<i>Acaca</i>	24.4	2.8	-3.39	0.997	1.97
<i>Acox1</i>	20.6	-1.1	-3.54	0.996	1.92
<i>Angptl3</i>	22.7	1.0	-2.78	0.988	2.29
<i>Angptl4</i>	28.5	6.8	-3.82	0.948	1.83
<i>Apob</i>	19.9	-1.7	-4.01	0.976	1.78
<i>Atf6</i>	26.3	4.6	-2.07	0.971	3.05
<i>Bdh1</i>	21.7	0.0	-3.58	0.997	1.90
<i>Cd36</i>	26.5	4.9	-1.27	0.906	6.15
<i>Chrebp</i>	21.9	0.3	-3.72	0.995	1.86
<i>Cideb</i>	21.6	0.0	-2.95	0.999	2.18
<i>Cpt1a</i>	23.0	1.3	-5.37	0.971	1.54
<i>Ddit3</i>	26.6	5.0	-2.80	0.992	2.28
<i>Dgat1</i>	25.7	3.9	-2.93	0.992	2.19
<i>Dgat2</i>	21.1	-0.5	-3.20	0.993	2.05
<i>Eif2ak3</i>	26.2	4.5	-2.13	0.981	2.94
<i>Fgfr2</i>	26.7	5.2	-2.86	0.974	2.24
<i>Gck</i>	24.1	2.5	-4.03	0.990	1.77
<i>Gpam</i>	21.7	0.1	-3.63	0.997	1.89
<i>Hngcs2</i>	19.5	-2.1	-3.47	0.998	1.94
<i>Hspa1b</i>	25.6	3.9	-2.62	0.980	2.41
<i>Insig1</i>	21.4	-0.2	-2.55	0.997	2.47
<i>Lipc</i>	21.5	-0.2	-2.26	0.981	2.77
<i>Mlycd</i>	24.5	2.8	-3.44	0.998	1.95
<i>Mttp</i>	23.2	1.5	-3.46	0.999	1.95
<i>Pc</i>	23.1	1.4	-3.87	0.995	1.81
<i>Pck1</i>	20.0	-1.8	-2.86	0.999	2.24
<i>Pdk4</i>	26.2	4.5	-3.20	0.991	2.05
<i>Plin2</i>	22.7	1.1	-3.39	0.996	1.97
<i>Pnpla2</i>	25.1	3.2	-3.20	0.994	2.05
<i>Ppara</i>	23.3	1.6	-3.84	0.996	1.82

Table A.1 (cont.)

Gene	Median Ct¹	Median ΔCt²	Slope³	(R²)⁴	Efficiency⁵
<i>Saa1</i>	23.9	2.2	-2.79	0.997	2.28
<i>Scd</i>	22.0	0.4	-3.36	0.963	1.99
<i>Slc27a2</i>	21.5	-0.1	-3.99	0.997	1.78
<i>Slc2a1</i>	27.3	5.6	-3.33	0.987	2.00
<i>Srebfl</i>	23.9	2.2	-3.23	0.997	2.04
<i>Ucp2</i>	25.8	4.1	-3.27	0.993	2.02
<i>Xbp1</i>	21.0	-0.7	-1.57	0.959	4.34

¹ The median is calculated considering all time points and all steers.

² The median of Δ Ct is calculated as [Ct gene – geometrical mean of Ct internal controls] for each time point and each steer.

³ Slope of the standard curve.

⁴ R² stands for the coefficient of determination of the standard curve.

⁵ Efficiency is calculated as [10(-1 / Slope)].

Table A.2 Accession number, sequence, amplicon size, reference and PCR efficiency of primers use to analyze gene expression by qPCR.

Accession #	Gene	Primer	Primers (5'-3')	Amplicon Size (bp)	Source
NM_030210.1	<i>Aacs</i>	F.230 R.329	TGGCGCTTGGGAATTACAAT CGTGAGTAGACGATTCCACTGAA	100	In this article
NM_133360.2	<i>Acaca</i>	F.2003 R.2087	CCTTCCTGCTCACACACTTCTG CTGCCGAGTCACCTTAAGTACATATT	85	
NM_015729	<i>Acox1</i>	F.436 R.537	GCCGTCGAGAAATCGAGAACT TCTTAACAGCCACCTCGTAACG	102	
NM_013913.3	<i>Angptl3</i>		AGGGCTTTGGAGGAGCAGCTAACC GCAGTCGGCAGGAAGGTCATCTTG		(250)
NM_020581.1	<i>Angptl4</i>		TTCCAACGCCACCCACTTACA ACCAAACCACCAGCCACCAGA		(251)
NM_009693.2	<i>Apob</i>		ATCTGACTGGGAGAGACAAGTAG CGATCAATAATCTCAGCAATAGTTC		(252)
NM_001081304.1	<i>Atf6</i>		TGCTAGGACTGGAGGCCAGGCTCAA CATGTCTATGAACCCAGCCTCGAAGT		(253)
NM_175177.3	<i>Bdh1</i>	F.402 R.201	GTCAGGCAGATGCGGCTAGT AGGAAGCCTTTTGAGTGCAGAT	100	In this article
NM_007643.3	<i>Cd36</i>	F.726 R.846	GAGGTCCTTACACATACAGAGTTCGTT ACAGACAGTGAAGGCTCAAAGATG	121	In this article
NM_021455.3	<i>Chrebp</i>	F.733 R.847	GCTTCTAGACCTGGACTGCTTCTT AGCATTGCCAACATAAGCATCTT	115	
NM_009894.2	<i>Cideb</i>		CCCCAAGAGTGGGATGTTGT GGGATTTTGCTTGTACACATCGA	100	In this article
NM_013495.1	<i>Cpt1a</i>	F.1722 R.1838	CCTGCATTCCCTTCCCATTG TTGCCCATGTCCTTGTAATGTG	117	
NM_007837.2	<i>Ddit3</i>		GCAAGGAAGAACTAGGAAACGGA CAATGTACCGTCTATGTGCAAGC		(254)
NM_010046.2	<i>Dgat1</i>	F.804 R.923	ACAACCTGACCTACCGAGATCTCT TCTCAAGAACTCGTCGTAGCAGAA	120	In this article

Table A.2 (cont.)

Accession #	Gene	Primer	Primers (5'-3')	Amplicon Size (bp)	Source
NM_026384.3	<i>Dgat2</i>	F.551	CCAAGAAAGGTGGCAGGAGAT	106	In this article
		R.656	GTCAGCAGGTTGTGTGTCTTCAC		
NM_010121.2	<i>Eif2ak3</i>		AACGGAAGGAGTCTGAAACTCAGT		(255)
			TTGGCTCAAAATCTGTTAGGTATCG		
NM_201601.2	<i>Fgfr2</i>		AAGGTTTACAGCGATGCCCA		(256)
			ACCACCATGCAGGCGATTAA		
NM_010292.4	<i>Gck</i>	F.389	TCTCTGACTTCCTGGACAAGCA	101	In this article
		R.489	GATGCCCTTGTCTATGTCTTCGT		
NM_008149.3	<i>Gpam</i>	F.725	TGGGTGTTACTAAAGCTCTTCAACA	100	In this article
		R.824	GCGGCAGGTTTCGTCTCAGT		
NM_008256.3	<i>Hmgcs2</i>	F.515	CACTGACATCGAGGGCATAGATAC	107	In this article
		R.621	CATAGCGACCATCCCAGTAGCT		
NM_010478.2	<i>Hspa1b</i>		AATTTAACAGTCAACGCAATTACC		(257)
			AACAGACTCTTTGCACTTGATAGC		
NM_153526.3	<i>Insig1</i>		AGGACGACAGTTAGCTATGGGTG		(258)
			CCCCCTTACCCGACTCTCACATAC		
NM_008280.2	<i>Lipc</i>	F.722	TTCTCGGAGCAAAGTTCACCTAA	110	In this article
		R.831	CCAGCCCTGTGATTCTTCCA		
NM_019966.2	<i>Mlycd</i>	F.903	CGCTGCCATCTTCTACTCCAT	105	In this article
		R.1007	AATTCCTTCTGCAGCTCCTTGA		
NM_008642.1	<i>Mttp</i>	F.234	GACAGCGTGGGCTACAAAATC	101	In this article
		R.334	GCTGTTATCGTGACTTGGATCACTT		
NM_008797.2	<i>Pc</i>	F.686	CGCATGAGTTCTCCAACACCTA	114	In this article
		R.799	TGTAATTCTCTTCCAACCTCATAGC		

Table A.2 (cont.)

Accession #	Gene	Primer	Primers (5'-3')	Amplicon Size (bp)	Source
NM_011044.2	<i>Pck1</i>	F.1453	CTGAAGGTGTCCCCCTTGTC	124	In this article
NM_013743.2	<i>Pdk4</i>	R.1576	GGGTCGTGCATGATGATCTTG CACATGCTCTTCGAACTCTTCAAG TGATTGTAAGGTCTTCTTTTCCCAAG		(259)
NM_007408.3	<i>Plin2</i>	F.725	AGCTGGAGATGGAAGCAAAAAA	101	In this article
NM_025802.2	<i>Pnpla2</i>	R.825	CCGAGAGCAGAGCTTGGTAGA		
NM_011144.3	<i>Ppara</i>	F.875	TGGAACCAAAGGACCTGATGAC	121	In this article
NM_009117.3	<i>Saa1</i>	R.995	AACAAGCGGATGGTGAAGGA ACTTCGCTATCCAGGCAGAA CAGACCAACCAAGTGTTGTGA GAAGGAAGCTAACTGGAAAACTC CAGGCCCCCAGCACAACTACT	117	(260)
NM_009127.3	<i>Scd</i>		TCCAGTGAGGTGGTGTGAAA TTATCTCTGGGGTGGGTTTG		
NM_011978.2	<i>Slc27a2</i>	F.1095	ACACCGCAGAAACCAAATGAC	100	In this article
NM_011400.2	<i>Slc2a1</i>	R.1194	CCCCAAATCTCTTGATGAAGTCTCT		
NM_011480.2	<i>Srebf1</i>	F.318	CCAGAAGGTTATTGAGGAGTTCTACAA	102	In this article
NM_011671	<i>Ucp2</i>	R.419	GCCACGGAGAGAGACCAAAG GTGAGCCTGACAAGCAATCA GGTGCCTACAGAGCAAGAGG		
NM_013842.2	<i>Xbp1</i>	F.881	GGCCTCTGGAAAGGGACTTC	103	
		R.983	TGGCTTTCAGGAGAGTATCTTTGA TCCGCAGCACTCAGACTATGT ATGCCCAAAGGATATCAGACTC		(261)

Table A.3 Sequencing results of PCR products from primers of genes designed for this experiment. Best hits using BLASTN (<http://www.ncbi.nlm.nih.gov>) are shown. Similar information for remaining genes was reported previously.

Gene	Sequence
<i>Aacs</i>	GGACTTTTGGGCTGAGTTCTGGAAGTTCAGTGGAAATCGTCTACTCACG
<i>Acaca</i>	GTACATATCTTAGTAAGGAATCAAATATGTACTTAAGGTGACTCGGCAGA
<i>Acox1</i>	
<i>Angptl3</i>	CTCAGGAGCACCCAGAAGTAACATCACTCAAAAGTTTTGTAGAACAGCAAGACAACAGCATAAGAGAACTCCT CCAGAGTGTGGAAGAACAGTATAAACAATTAAGTCAACAGCACATGCAGATAAAAGAAATAGAAAAGCAGCTC AGAAAGACTGGTATTCAAGAACCCTCAGAAAATTCTTTCTTCTAAATCAAGAGCACCAAGAACTACTCCCCCT CTTCAACTGAACGAAACAGAAAATACAGAACAAGATGACCTTCCTGCCGACTGC
<i>Angptl4</i>	GGGAGCGGCACAGTGGACTTTTCCAGATCCAGCCTCTGGGGTCTCCACCATTTTTGGTCAACTGTGAGATGACTT CAGATGGAGGCTGGACAGTGATTCAGAGACGCCTGAACGGCTCTGTGGACTTCAACCAGTCCTGGGAAGCCTAC AAGGATGGCTTCGGAGATCCCCAAGGCGAGTTCTGGCTGGGCCTGGAAAAGATGCACAGCATCACAGGGAACC GAGGAAGCCAATTGGCTGTGCAGCTCCAGGACTGGGATGGCANTGCCNAATTGCTCCAATTTCCCATCCATTG GGGGGTGAGGA
<i>Apob</i>	AAATTATAGAATTACAGATAATGATGTACTAATTGCCATAGATAGTGCCAAAATCAACTTCAATGAAAAACTCT CTCAACTTGAGACATACGCGATATAATTTGATCAGTATATTAAAGATAATTATGATCCACATGACTTAAAAAGA ACTATTGCTGAGATTATTGATCGAA
<i>Atf6</i>	AGGAGAATGGCTCCCTGAAGCGACAGCTGGACGAGGTGGTGTGTCAGAGAACCAGAGGCTCAAAGTCCCAAGTCC AAAGCGAAGAGCTGTCTGTGTGATGATAGTATTAGCATTTATAATGCTGAACTATGGGCCCATGAGCATGCTGG AGCAAGAATCCCGAAGAGTGAAACCTAGTGTGAGCCCTGCCAATCAGAGGAGGCATCTCTTGGAATTTTCAGCA AAAGAAGTTAAAGACACATCAGATGGTGACAACCAGAAAGACAGTTACAGCTATGATCACTCTGTGTCCAATGA CAAAGCTTTAATGGTGCTAAGTGAAGAGCCATTGCTTTATATGCCTCCACCTCCATGTCAACCCCTGATTAACAC AACAGAGTCTCTCAGGTTGAACCATGAACTTCGAGGCTGGGTTTCATAGACATGA
<i>Bdh1</i>	GATTTGGGTTCTCACTGGCCAAGCATCTGCACTCAAAAGGCTTCCT

Table A.3 (cont.)

Gene	Sequence
<i>Cd36</i>	CCATCTTTGAGCCTTCACTGTCTGT
<i>Chrebp</i>	CTCTTCACCATGACACAGCCCAGTCCTTCGTCCCTGCAGCTGCCCCCAGAAGAATGCTTATGTTGGCAATGCTAA
<i>Cideb</i>	AGCAAGGACATCGCCCCGCATCACCTTCGATGTGTACAAGCAAAATCCCC
<i>Cpt1a</i>	CTGATCAGAAGTGCCGGACGAGTCCCGATGCCTTCATCCAGCTGGCACTGCAGCTCGCACATTACAAGGACATGG GCAAA
<i>Ddit3</i>	CCGGCCTGGGAGCACGCATGAAGGAGAAGGAGCAGGAGAACGAGCGGAAAGTGGCACAGCTAGCTGAAGAGA ACGAGCGGCTCAAGCAGGAAATCGAGCGCCTGACCAGGGAGGTGGAGACCACACGGCGGGCTCTGATCGACCG CATGGTCAGCCTGCACCAAGCATGAACAGTGGGCATCACCTCCTGTCTGTCTCTCCGGAAGTGTACCCAGCACCA TCGCGCCAGCGCCAAGCATGTGACCCTGCACTGCACTGCACATGCTGAGGAGGGGACTGAGGGTAGACCAGGAG AGGGCTCGGCTTGCACATAGACGGTACATTG
<i>Dgat1</i>	TCAACTTTCCTCGGTCCCCCGAATACGAAAGCGCTTTCTGCTACGACGAGTTCTTGAGA
<i>Dgat2</i>	GTGTGCGGCTACTTCCGAGACTACTTTCCCATCCAGCTGGTGAAGACACACAACCTGCTGAC
<i>Eif2ak3</i>	GTGCCGATGTCAGTGACAACAGCTGGAATGACATGAAGTACTCAGGATACGTATCCCGATACCTAACAGATTTTG AGCCAA
<i>Fgfr2</i>	ACGGCAGTAAATACGGGCCTGATGGGCTGCCCTACCTCAAGGTCCTGAAGCACTCGGGGATAAATAGCTCCAAT GCAGAAGTGCTGGCTCTGTTCAATGTGACGGAGATGGATGCTGGGGCAATATATATGTAAGGTCTCCAATTATAT AGGGCAGGCCAACCAGTCTGCCTGGCTCACTGTCCTGCCCAAACAGCAAGCGCCTGTGAGAGAGAAGGAGATCA CGGCTTCCCCAGATTATCTGGAGATAGCTATTTACTGCATAGGGGTCTTCTTAATCGCCTGCATGGTG
<i>Gck</i>	CCCCGTGGCTTCACCTTCTCCTTCCCGTTAAGGCACGAAGACATAGACAAGGGCATC
<i>Gpam</i>	AGGGTCAACTCGAGATGGTCAAGGCTGCAACTGAGACGAACCTGCCGC
<i>Hmgcs2</i>	GCGGCACAGCCTCCCTCTTCAATGCTGCCAACTGGATGGAGTCCAGCTACTGGGATGGTCGCTATG
<i>Hspa1b</i>	CCATGAAGAAGACTTTAAATAACCTTGACAGTAATCGGTGCCCAAGCAGCTATCAAGTGCAAAGAGTCTGTT

Table A.3 (cont.)

Gene	Sequence
<i>Insig1</i>	CACATGGTTCAGAGTGGAAGCAGGATGTAGAGACACTGGTCCTGGGTGTGATGAAGATGATCTTTTTCTCAATG TTCCATTTAGGCTGGGCTGACGATAAATGACTCCTAAAGACATGTTCACTTAGTCTAGACTAGCAAATGGAGGC AAGGACTGCTTACCTAAAAGTCTTACCTTGCTCCCCCACCCTCACACCTGTCTTCTTTGGAACATTCCATCCCAG GCTGTATGTGAGAGTCGGGGTAAGGGGG
<i>Lipc</i>	CGTCTCAGGGTTCGCAGGCAGCTCCATGGACGGGAAGAACAAGATTGGAAGAATCACAGGGC
<i>Mlycd</i>	GAGCTCGGGACCTTCCTCATAAAGAGAGTGGTCAAGGAGCTGCAGAAGGAATT
<i>Mtp</i>	GGAGGAATCCTGATGGTGATGATGATCAAGTGATCCAAGTCACGATAACAGC
<i>Pc</i>	GCCGCCTACGGAGGTGGGGGCCGCGGCATGCGGGTCGTGCATAGCTATGAGGAGTTGGAAGAGAATTACA
<i>Pck1</i>	GGAGCAGCCATGAGATCTGAGGCCACAGCTGCTGCAGAACACAAGGGCAAGATCATCATGCACGACCC
<i>Pdk4</i>	GAGCATCAAGAAAACCGTCCTTCCTTGACCCCAGTAGAGGCCACTGTCTGTTGGGAAAAGAAGACCTTACAAT CAA
<i>Plin2</i>	AAGCCGAGCAACTATGAACGGCTGGAGTCCCTGTCTACCAAGCTCTGCTCTCGGG
<i>Pnpla2</i>	GCGCCTGGCAACGGCCATGATGGTGCCCTATACTCTGCCGCTGGAGAGTGCAGTGTCTTCACCATCCGCTTGTT
<i>Ppara</i>	
<i>Saa1</i>	ATGCTGCTCAAAGGGGTCCCGGGGGAGTCTGGGCTGCTGAGAAAATCAGTGATGGAAGAGAGGCCTTTCAGGA ATTCTTCGGCAGAGGACATGAGGACACCATTGCTGACCAGGAAGCCAACAGACATGGCCGCAGTGGCAAAGAC CCCAATTACTACAGACCTCCTGGACTGCCTGACAAATACTGAGCGTCCTCCTATTAGCTCAGTAGGTTGTGCTGG GGGCCTGA
<i>Scd</i>	CTCAGCCAAGGTGCCTCTTAGCCACTGAATTGCTATGTTATCCTTTCTCTTGTAACAAACCCACCCAGAGATAA A
<i>Slc27a2</i>	GGAAATGGCTTACGAGGAGATGTGTGGAGAGAGTTCATCAAGAGATTTGGGG
<i>Slc2a1</i>	GGAGAGCCCATCCCATCCACCACACTCACCACGCTTTGGTCTCTCTCCGTGGC
<i>Srebfl</i>	GGTCAAGAATCTGGAAATTGCAGAGGCTGCACTGGCCCGATGGCACCTCTTGCTCTGTAGGCACCAAT
<i>Ucp2</i>	
<i>Xbp1</i>	TCCCCAGAACATCTTCCCATGGACTCTGACACTGTTGCCTCTTCAGATTCTGAGTCTGATATCCTTTTGGGCAT

Table A.4 Sequencing results of genes using BLASTN from NCBI against nucleotide collection (nr / nt) with total score.

Gene	Best hit in NCBI	Score
<i>Aacs</i>	Mus musculus acetoacetyl-CoA synthetase (Aacs), mRNA	80.1
<i>Acaca</i>	Mus musculus acetyl-Coenzyme A carboxylase alpha (Acaca), mRNA	89.1
<i>Acox1</i>	Mus musculus acyl-Coenzyme A oxidase 1, palmitoyl (Acox1), mRNA.	80.1
<i>Angptl3</i>	Mus musculus angiopoietin-like 3 (Angptl3), mRNA	89.1
<i>Angptl4</i>	Mus musculus angiopoietin-like 4 (Angptl4), mRNA	78.3
<i>Apob</i>	Mus musculus chromosome 12 genomic contig, strain C57BL/6J	89.1
<i>Atf6</i>	Mus musculus activating transcription factor 6 (Atf6), mRNA	94.5
<i>Bdh1</i>	Mus musculus 3-hydroxybutyrate dehydrogenase, type 1 (Bdh1),mRNA	78.3
<i>Cd36</i>	Mus musculus CD36 antigen (Cd36), mRNA	94.5
<i>Chrebp</i>	Mus musculus MLX interacting protein-like (Mlxipl), Mrna	87.3
<i>Cideb</i>	Mus musculus cell death-inducing DNA fragmentation factor, alpha subunit-like effector B (Cideb), mRNA	80.1
<i>Cpt1a</i>	Mus musculus carnitine palmitoyltransferase 1a, liver (Cpt1a),	78.3
<i>Ddit3</i>	Mus musculus DNA-damage inducible transcript 3 (Ddit3), mRNA	85.5
<i>Dgat1</i>	Mus musculus diacylglycerol O-acyltransferase 1 (Dgat1), mRNA	89.1
<i>Dgat2</i>	Mus musculus diacylglycerol O-acyltransferase 2 (Dgat2), mRNA	81.9
<i>Eif2ak3</i>	Mus musculus eukaryotic translation initiation factor 2 alpha kinase 3 (Eif2ak3), mRNA	81.9
<i>Fgfr2</i>	Mus musculus fibroblast growth factor receptor 2 (Fgfr2), transcript variant 2, mRNA	74.7
<i>Gck</i>	Mus musculus glucokinase (Gck), mRNA	83.7
<i>Gpam</i>	Mus musculus glycerol-3-phosphate acyltransferase, mitochondrial (Gpam), mRNA	81.9
<i>Hmgcs2</i>	Mus musculus 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (Hmgcs2), mRNA	85.5
<i>Hspa1b</i>	Mus musculus heat shock protein 1B (Hspa1b), mRNA	89.1
<i>Insig1</i>	Mus musculus insulin induced gene 1 (Insig1), mRNA	87.3

Table A.4 (cont.)

Gene	Best hit in NCBI	Score
<i>Lipc</i>	Mus musculus lipase, hepatic (<i>Lipc</i>), mRNA	80.1
<i>Mlycd</i>	Mus musculus malonyl-CoA decarboxylase (<i>Mlycd</i>), mRNA	80.1
<i>Mttp</i>	Mus musculus microsomal triglyceride transfer protein (<i>Mttp</i>), mRNA	83.7
<i>Pc</i>	Mus musculus pyruvate carboxylase (<i>Pcx</i>), mRNA	89.1
<i>Pck1</i>	Mus musculus phosphoenolpyruvate carboxykinase 1, cytosolic (<i>Pck1</i>), mRNA	76.5
<i>Pdk4</i>	Mus musculus pyruvate dehydrogenase kinase, isoenzyme 4 (<i>Pdk4</i>), mRNA	92.7
<i>Plin2</i>	Mus musculus adipose differentiation related protein (<i>Adfp</i>), mRNA	80.1
<i>Pnpla2</i>	Mus musculus patatin-like phospholipase domain containing 2 (<i>Pnpla2</i>) mRNA	78.3
<i>Ppara</i>	Mus musculus peroxisome proliferator activated receptor alpha (<i>Ppara</i>)	76.5
<i>Saa1</i>	Mus musculus serum amyloid A 1 (<i>Saa1</i>), mRNA	85.5
<i>Scd</i>	Mus musculus stearoyl-Coenzyme A desaturase 1 (<i>Scd1</i>), mRNA	74.7
<i>Slc27a2</i>	Mus musculus solute carrier family 27 (fatty acid transporter), member 2 (<i>Slc27a2</i>), mRNA	85.5
<i>Slc2a1</i>	Mus musculus solute carrier family 2 (facilitated glucose transporter), member 1 (<i>Slc2a1</i>), mRNA	87.3
<i>Srebf1</i>	Mus musculus sterol regulatory element binding factor 1 (<i>Srebf1</i>), mRNA	74.7
<i>Ucp2</i>	Mus musculus uncoupling protein 2 (mitochondrial, proton carrier)	81.7
<i>Xbp1</i>	Mus musculus X-box binding protein 1 (<i>Xbp1</i>), mRNA	81.9

Table A.5 *In vitro* studies of the *trans*-10, *cis*-12-CLA included in the literature review.

Reference	Subjects	Dose	Duration	Results
Bretillon et al. (1999) (111)	Rat liver microsomes	20 to 120 nmol/L	15 min	<i>Trans</i> -10, <i>cis</i> -12-CLA significantly inhibited the conversion of stearic acid and the $\Delta 6$ desaturation of linoleic acid at the highest inhibitor/substrate ratio.
Yotsumoto et al. (1999) (146)	HepG2 cells	10 μ mol/L	24 hrs	<i>Trans</i> -10, <i>cis</i> -12-CLA inhibited cellular triglyceride synthesis and reduced apoB secretion.
Lin et al. (2001) (144)	HepG2 cells	1 mmol/L	5 hrs	<i>Trans</i> -10, <i>cis</i> -12-CLA suppressed TG secretion in spite of the fact that it increased 4-fold the cellular TG content.
Choi et al. (2001) (109)	HepG2 cells	45 μ mol/L	48 hrs	Human SCD activity was \downarrow by the <i>trans</i> -10, <i>cis</i> -12-CLA directly, without changes in SCD gene expression, suggesting that this isomer regulates human SCD activity mainly by a posttranslational mechanism.
Clément et al. (2002) (33)	COS-1 cells	10 to 200 μ mol/L	36 hrs	<i>Trans</i> -10, <i>cis</i> -12-CLA and <i>cis</i> -9, <i>trans</i> -11-CLA were equally efficient at activating PPAR α , β/σ and γ and inhibiting liver-X-receptor. Thus, the specific effect of <i>Trans</i> -10, <i>cis</i> -12-CLA is unlikely to result from direct interaction with these nuclear receptors.
Eder et al. (2002) (108)	HepG2 cells	1 to 100 μ mol/L	0.5 or 24 hrs	Cells had a markedly lower ratio of MUFA to SFA in lipids than control cells, suggesting that <i>trans</i> -10, <i>cis</i> -12-CLA suppresses $\Delta 9$ -, $\Delta 6$ - and $\Delta 5$ -desaturation and has significant effects on the metabolism of essential fatty acids in HepG2 cells.
Yamasaki et al. (2005) (50)	Rat hepatoma cells	0 to 10 μ mol/L	72 hrs	1 μ mol/L <i>Trans</i> -10, <i>cis</i> -12-CLA demonstrated significant cytotoxic effects on rat hepatoma cells. It also induced mitochondria-related apoptosis accompanied by lysosomal destabilization in rat hepatoma cells.
Wei et al. (2007) (262)	Rat hepatoma cells	80 μ mol/L	3, 6 or 16 hrs	<i>Trans</i> -10, <i>cis</i> -12-CLA reduced SCD1 activity and increased the SFA composition of total lipids following incubations with palmitate. Co-supplementation of palmitate with <i>Trans</i> -10, <i>cis</i> -12-CLA resulted in a significant increase ER stress response genes and apoptosis.

Table A.6 *In vivo* studies of the *trans*-10, *cis*-12-CLA included in the literature review.

Reference	Subjects	Dose	Duration	Results
de Deckere et al. (1999) (85)	Male F ₁ B hybrid hamsters	6.6 g/kg diet	8 wks	<i>Trans</i> -10, <i>cis</i> -12-CLA and the CLA mix decreased fasting values of LDL- and HDL-cholesterol, increased VLDL-TG and decreased epididymal fat pad weights. <i>Trans</i> -10, <i>cis</i> -12-CLA increased liver weight but not liver lipids. Liver histology revealed that increased weight was due to hypertrophy. Data suggested that <i>Trans</i> -10, <i>cis</i> -12-CLA stimulated the oxidation of all C ₁₈ PUFAs.
Clément et al. (2002) (33)	Female C57B1/6J mice	0.4% (w/w)	4 wks	<i>Trans</i> -10, <i>cis</i> -12-CLA reduced adipose tissue and triggered a massive enlargement of the liver (3.1 fold increase), which displayed the typical features of a fatty liver: pale color and accumulation of intracellular lipids. <i>Trans</i> -10, <i>cis</i> -12-CLA-induced hyperinsulinemia may trigger liver steatosis by inducing both fatty acid uptake and lipogenesis.
Degrace et al. (2003) (30)	7-wk-old male C57BL/6j mice	10 g	4 wks	This study demonstrated that the steatosis accompanying the fat loss induced by <i>Trans</i> -10, <i>cis</i> -12-CLA. The 3-fold decrease in plasma TG contents and the induction of mRNA expression of LDL receptors concomitantly observed in <i>Trans</i> -10, <i>cis</i> -12-CLA-fed mice suggested an increase in the lipoprotein clearance at the level of the liver itself.
Warren et al. (2003) (113)	8-wk-old female mice	0.5%	8 wks	Body and retroperitoneal adipose tissue weights were significantly lower and liver weights were significantly greater (100%) in the <i>Trans</i> -10, <i>cis</i> -12-CLA group. Livers from animals in the <i>Trans</i> -10, <i>cis</i> -12-CLA group contained 5 times more lipids than in the control group. <i>Trans</i> -10, <i>cis</i> -12-CLA decreased PPAR α mRNA, suggesting that PPAR α did not mediate the effects of <i>Trans</i> -10, <i>cis</i> -12-CLA on body composition.

Table A.6 (cont.)

Reference	Subjects	Dose	Duration	Results
Kelley et al. (2004) (25)	8-wk-old femal C57BL/6N mice	0.5%	8 wks	Livers from animals fed <i>Trans</i> -10, <i>cis</i> -12-CLA diet contained 4 times more lipids than those of the control group; this was mainly due to an increase in the TG fractions. <i>Trans</i> -10, <i>cis</i> -12-CLA caused an increase in the weight percentage of 18:1n-9, possibly due to an increased activity of SCD.
Zabala et al. (2004) (114)	9-wk-old male hamsters	5 g	6 wks	<i>Trans</i> -10, <i>cis</i> -12-CLA produced significantly greater liver weight, but also significantly decreased liver fat accumulation. No changes in mRNA levels of Srebp-1a, Srebp-1c and lipogenic enzymes, or in the activities of these enzymes, were observed.
Sanders et al. (2004) (89)	Growing female obese Zucker rats	1.5 g/kg BW	3 wks	Average daily gain was reduced 44% by the <i>Trans</i> -10, <i>cis</i> -12-CLA. <i>Trans</i> -10, <i>cis</i> -12-CLA increased liver lipid content by 33% without effects on liver weight.
Degrace et al. (2004) (31)	7-wk-old male C57BL/6J mice	1%	4 wks	Mice fed the <i>Trans</i> -10, <i>cis</i> -12-CLA-fed had enlarged livers and hypotrophied periepididymal adipose tissues. CPT1 and carnitine-dependent palmitate oxidation activities were also significantly greater in <i>Trans</i> -10, <i>cis</i> -12-CLA-fed mice than in the two other groups, indicating that FA oxidation capacities were increased in mice fed <i>Trans</i> -10, <i>cis</i> -12-CLA.
House et al. (2005) (248)	Adult male mice	1%	2 wks	<i>Trans</i> -10, <i>cis</i> -12-CLA reduced weights of adipose tissues and increased liver weight by 34%.

Table A.6 (cont.)

Reference	Subjects	Dose	Duration	Results
Viswanadha et al. (2006) (24)	4-wk-old CD-1 mice	0.15% or 0.30%	6 wks	Liver weight of male and female mice increased in response to <i>Trans</i> -10, <i>cis</i> -12-CLA in a dose-dependent manner and the increase was accompanied by an increase in total FA content. Unlike adipose tissue, overall hepatic SCD mRNA expression was not affected by <i>Trans</i> -10, <i>cis</i> -12-CLA.
Gudbrandsen et al. (2009) (36)	5-wk-old male obese Zucker rats	1%	10 d	<i>Trans</i> -10, <i>cis</i> -12-CLA reduced the liver TG content without improving the overall adiposity, enhanced hepatic mitochondrial and peroxisomal β oxidation by stimulation of PPAR α . The reduced hepatic TG content may be partly due to lower activity of SCD.
Halade et al. (2009) (48)	11-m-old female C57B1/6J mice	0.5%	0.5 y	<i>Trans</i> -10, <i>cis</i> -12-CLA was found to reduce the fat mass and to increase the lean mass but significantly contributed to increase insulin resistance and liver hypertrophy.
Ashwell et al. (2010) (32)	9-wk-old male mice	1%	2 wks	The mice fed <i>Trans</i> -10, <i>cis</i> -12-CLA had 62.5% more fat in the livers with a 33% increase in liver weight.

Table A.7 Clinical studies of the *trans*-10, *cis*-12-CLA included in the literature review.

Reference	Subjects	Dose	Duration	Results
Berven et al. (2000) (117)	55 overweight or obese > 18 years	3.4 g/d of CLA mixture	12 wks	A dose of 3.4 g of CLA daily for 12 weeks is well tolerated in the population studied. No clinically significant changes in blood parameters or vital signs occurred. The use of CLA does not impair liver functions in humans.
Blankson et al. (2000) (45)	47 overweight or obese > 18 years	1.7, 3.4, 5.1 or 6.8 g/d of CLA mixture (CLA1:CLA2 = 1:1)	12 wks	CLA may reduce body fat mass (BFM) in humans and no addition effect on BFM is achieved with doses > 3.4 g CLA/d.
Noone et al. (2002) (194)	51	3 g/d of CLA mixture (CLA1:CLA2 = 1:1)	8 wks	CLA significantly reduced fasting plasma TG concentrations. No significant effect on LDL-cholesterol, HDL-lipid-protein composition or reverse cholesterol transport occurred.
Kamphuis et al. (2003) (263)	54 overweight 20-50 years	1.8 or 3.6 g/d of CLA mixture (CLA1:CLA2 = 1:1)	13 wks	The regain of fat-free mass was favorably, dose-independently affected by a 13-week consumption of 1.8 or 3.6 g CLA/day and consequently increased the resting metabolic rate. However, it did not result in improved body weight maintenance after weight loss.
Malpuech-Brugere et al. (2004) (102)	81 overweight healthy middle-aged	1.5 or 3 g/d of CLA2	18 wks	A daily consumption of a drinkable dairy product containing up to 3 g of CLA isomers for 18 weeks had no statistically significant effect on body composition in overweight, middle-aged men and women.

Table A.7 (cont.)

Reference	Subjects	Dose	Duration	Results
Whigham et al. (2004) (120)	50 overweight or obese 18-50 years	6 g/d of CLA mixture (CLA1:CLA2 \approx 1:1)	1 y	Body composition did not differ between groups. Laboratory tests showed no adverse effects of CLA. The study concluded that CLA as Clarinol TM is safe for use in obese humans for at least one year.
Gaullier et al. (2005) (264)	125 Mean age: 45-48	3.4 g/d of CLA mixture (CLA1:CLA2 \approx 1:1)	1 y	Aspartate amino transferase but not alanine amino transferase, increased significantly. Plasma total cholesterol and LDL cholesterol were reduced, whereas HDL cholesterol and TGs were unchanged. It confirms that CLA decreases BFM in overweight humans and may help maintain initial reductions in BFM and weight in the long term.
Iwata et al. (2007) (119)	60 healthy overweight 25-60 years	3.4 or 6.8 g/d of CLA mixture (CLA1:CLA2 = 1:1)	12 wks	There was a slight increase in the level of liver enzymes in 6.8 g/d CLA group at 12 weeks. But these changes were within the normal range. The study also indicated that the use of CLA does not impair liver function in overweight male humans whose blood parameters are within the normal range.
Wanders et al. (2010) (64)	20 healthy BMI < 30 kg/m ² 18-60 years	14.6 g CLA1 + 4.7 g CLA2	3 wks	A daily intake of 19.3 g CLA for 3 weeks does not produce clinically relevant effects on markers of liver and kidney function in healthy volunteers.
Venkatramanan et al. (2010) (116)	11 moderately overweight 30-60 years	Milk enriched with 1.3 g/d of CLA mixture	24 wks	Supplementation with CLA-enriched milk did not significantly affect liver function or body weight.

Curriculum Vitae

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Education

University of Illinois at Urbana-Champaign 08/2009-05/2011

M.S. of Nutritional Sciences, GPA: 3.83/4.0

Thesis: Hepatic gene networks due to the trans-10, cis-12-conjugated linoleic acid in growing male mice

The University of Hong Kong 09/2006-07/2009

B.S. of Food and Nutritional Sciences

Professional and Research Experience

Research Assistant, University of Illinois at Urbana Champaign 08/2009-05/2011

- Leading a project investigating the effects of t10, c12-CLA intake on hepatic gene expression in growing male mice
- Working independently with minimum supervision and successfully performed Primer Design & Testing, Internal Control Genes Selection, Gene Network Analysis, RT-PCR, Gel Electrophoresis, DNA/RNA Extraction & Purification, **Excellent Pipetting Skill**
- Strong ability to carry out scientific data analyses, interpretation and visualization in the form of graphs, charts and tables
- Have presented 5 most recently published and top-tier papers in one-hour seminars

Intern, Product Development Department, Nestlé's R&D, Beijing 07/2010-09/2010

Developing a signature taste for Nestlé dairy products, aiming to expand product line and improve quality/cost

- Screened and Evaluated a total of 40 flavors and more than 20 stabilizers and emulsifiers for dairy products
- Performed bench top and pilot plant experiments and Designed triangle tests/informal tasting test for innovative formula, quality improvement and ingredient substitution
- Collected data and Conducted analyses on industry/market, technology, competitors and

risks; Prepared reports, graphs and charts; Presented directly to Department Manager
- Successfully Offered a full-time position as Product Development Trainee for 2011 (the only intern to receive the honor)

Intern, USDA Human Nutrition Research Center on Aging, Boston 06/2008-08/2008

- Quickly mastered Western blotting, Cell Culture, Western Blot, BCA Protein Concentration Assay, Laser-Capture Microdissection in one week, generating reliable data for curcumin research

- Co-authored "Regulation of LPS-induced tissue factor expression in human monocytic THP-1 cells by curcumin"*

*http://www.fasebj.org/cgi/content/meeting_abstract/23/1_MeetingAbstracts/717.2

Research Student, Genetics Lab, The University of Hong Kong 09/2007-07/2008

- Undergraduate Research Student which was only offered to the students with excellent academic performance

- Successfully carried out a project on Campylobacter and Presented findings in a seminar to entire faculty of science

- Wrote a 50-page dissertation: **Surveying of Campylobacter jejuni in Local Poultry Farm with Phylogenetic Analysis on gyrB Gene**

- Learned and Performed DNA Extraction, qPCR, Gel Electrophoresis, Immunoassay Identification, Gram Stain Identification

Honors and Awards

2011 Nutrition Policy and Regulatory Affairs Summit, Washington, DC, Sponsored by Kraft Foods and University of Illinois Urbana Champaign 05/2011

The First Price Scholarship in the High School Entrance Examination 09/2002

The First Price of National English Proficiency Contest for Secondary Students 12/2001

Skills

Language: English, Chinese

Computer: MS Office, Advanced Excel, Adobe Suite, SAS, SigmaPlot,

DNASTARLasergene.7.1, Molecular Evolutionary Genetics Analysis, Primer
Premier, Primer Express 3, Sequence Detection System Software, geNorm,
Ingenuity Pathway Analysis

Projects

Graduate Study Proposal: Effect of High Pressure Heat Treatment and Polysaccharide
Addition on Beta-Lactoglobulin (Allergy Project)

Individual Nutritional Topic: Bioactive Compounds Extracted From Juice-Making Waste
Inhibit Human Pancreatic Cancer Cells

HACCP Project: Enjoy Sports, Drink O' Beer

Evidence Based Practice: Does Caffeine Increase the Risk for Osteoporosis in Postmenopausal
Women?